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CHARACTERIZATION OF CD8$^+$ T-CELL POPULATIONS OF THE HUMAN PERIPHERAL BLOOD

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‘Make not your thoughts your prisons’

– William Shakespeare

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Doing a PhD is may be one of the most difficult challenges one can face in life. Not only because of the responsibility, the amount of work, the disappointments, the pressure, etc., but probably because of the combination of all these factors. It is quite difficult to put in words and everyone I know who has a PhD felt the same way. Hence, the support I had from some people was fundamental for me to reach the day when I wrote the last page of this manuscript. I own them these words… and much more.

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ABBREVIATIONS

APC	Antigen Presenting Cell
BCL-2	B-Cell Lymphoma-2
CD	Cluster of Differentiation
CMV	Cytomegalovirus
CTL	Cytotoxic T-Lymphocyte
DC	Dendritic Cell
EBV	Epstein Barr Virus
HCV	Hepatitis C Virus
HEV	High Endothelial Venules
HIV	Human Immunodeficiency Virus
IFN	Interferon
IL	Interleukin
LFA	Lymphocyte Function-Associated Antigen
LN	Lymph Nodes
LTA	Lymphotoxin α
MIP	Macrophage Inflammatory Protein
MHC	Major Histocompatibility Complex
PCR	Polymerase Chain Reaction
PTK	Protein Tyrosine Kinase
RANTES	Regulated Upon Activation, Normally T-Expressed, and Presumably Secreted
RT	Reverse Transcription
T_{CM}	Central Memory T Cells
TCR	T-Cell Receptor
T_{EM}	Effector Memory T Cells
T_{EMRA}	Effector Memory CD45RA$^+$ T Cells
TGF	Transforming Growth Factor
T_H	T Helper Cells
T_N	Naïve T Cells
TNF	Tumor Necrosis Factor
γc	Common γ Chain

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RESUME

Après stimulation antigénique, les lymphocytes T $CD8^+$ naïves subissent plusieurs modifications, comme l'expression des molécules de surface. Chez l'homme, l'association du CCR7, CD45RA, CD27 et CD28 est fréquemment utilisée pour discriminer de façon reproductible des sous-populations de cellules T $CD8^+$ fonctionnellement différentes. Néanmoins, la description de ces populations reste incomplète, puisque plusieurs études ont utilisé des associations différentes et limitées de molécules de surface. En conséquence, certaines sous-populations de cellules T $CD8^+$ n'ont pas encore été établies, en particulier dans les compartiments $CCR7^-CD45RA^+$ et $CCR7^-CD45R0^+$. De plus, les voies de différenciation de ces sous-populations ainsi que leurs rôles respectifs ne sont pas encore définis.

L'objet de ce travail était de définir une corrélation précise et prévisible entre un phénotype de surface donné et des propriétés fonctionnelles des cellules T $CD8^+$. Nous avons associé les niveaux d'expression de CCR7, CD45RA, CD27 et CD28 pour subdiviser les cellules T $CD8^+$ en quatorze sous-types cellulaires différents. Ces populations ont été isolées et l'expression génique de 18 gènes a été étudiée simultanément sur des cellules uniques, par une nouvelle méthode de RT-PCR multiplex que nous avons développée. Nos résultats démontrent que les différentes populations présentent des profils d'expression génique caractéristiques et distincts, reproductibles entre différents donneurs. L'expression de CD45RA est nécessaire pour identifier les cellules naïves, mais ne discrimine pas les différentes populations de cellules qui ont déjà rencontré l'antigène. Par contre, les profils d'expression génique des cellules T $CD8^+$ $CCR7^-$ montrent une importante corrélation avec les niveaux d'expression de CD27 ainsi qu'avec la co-expression CD27/CD28. Une hiérarchie d'activation a été établie de la façon suivante : naïve < $CD27^{high}$ < $CD27^+CD28^+$ < $CD28^+CD27^-$ < $CD27^+CD28^-$ < $CD27^-CD28^-$. De plus, nous montrons que les cellules $CD45RA^+$ et $CD45RA^-$ appartenant à ces sous-populations ont des profiles d'expression génique identiques, au niveau qualitative et quantitative. Par ailleurs, nous avons identifié des sous-populations mineures avec des caractéristiques d'activation récente parmi les compartiments $CD45RA^+$ et $CD45RA^-$. Ces résultats suggèrent que la différentiation des cellules naïves T $CD8^+$ en cellules effectrices n'oblige pas à une perte d'expression de CD45RA. Nous avons, donc, décrit des nouvelles populations T $CD8^+$ et établis une corrélation entre le phénotype de surface et les fonctions cellulaires, ce qui permet d'identifier des populations cellulaires homogènes.

SUMMARY

Following antigenic challenge, naïve $CD8^+$ T lymphocytes undergo several changes, including the expression of cell-surface molecules. In humans, the association of CCR7, CD45RA, CD27 and CD28 is widely used to discriminate a reproducible set of functionally different subpopulations of $CD8^+$ T cells. However, the prevailing data concerning the description of these subsets remains fragmentary, since a multitude of studies used a different and limited set of surface markers. Hence, some $CD8^+$ T-cell subsets are still not clearly established, especially within the $CCR7^-CD45RA^+$ and $CCR7^-CD45R0^+$ compartments, and the correspondent differential roles and lineage relationships remain undisclosed.

The present study aims to define a predictable and precise correlation between particular cell surface markers and $CD8^+$ T-cell functional properties. We associated CCR7, CD45RA, CD27 and CD28 expression levels to subdivide $CD8^+$ T cells into fourteen different cell types. These populations were further isolated and gene expression of 18 genes was assessed, simultaneously, in single-cells by a novel multiplex RT-PCR method we developed. Our results demonstrate that the different subpopulations display distinct and characteristic gene co-expression patterns, reproducible between donors. CD45RA expression is required to define the naïve subset, but does not discriminate functionally different populations of primed cells. In contrast, gene expression profiles of $CCR7^-CD8^+$ T cells correlate significantly to CD27 expression levels and CD27/CD28 co-expression, and a hierarchy of activation stages could be established as follows: naïve < $CD27^{high}$ < $CD27^+CD28^+$ < $CD28^+CD27^-$ < $CD27^+CD28^-$ < $CD27^-CD28^-$. Surprisingly, we found that $CD45RA^+$ and $CD45RA^-$ cells of each of these subsets had the same gene expression patterns at both qualitative and quantitative level. Importantly, we identified minor subsets displaying characteristics of recent activation that could be found in both $CD45RA^+$ and $CD45RA^-$ compartments. These findings strongly suggest that differentiation of naïve $CD8^+$ T cells into effectors does not necessarily imply CD45RA downregulation. Furthermore, they describe novel $CD8^+$ T cell subsets and establish a correlation between surface phenotype and cell function, which helped to identify homogeneous populations.

INTRODUCTION

1. The immune system: an overview

Every living being is continuously threatened by environmental aggressions, including other organisms. These threats have acted as selective pressures among evolution driving species to evolve several defensive mechanisms that allow them to escape and survive such dangers. In response to invading pathogens, eukaryotes have evolved an elaborate protective apparatus generally known as the immune system. The word “immune” comes from the Latin immunis, meaning “exempt”. In this way, an organism that is immune to a specific infecting agent is able to remain free from infection by that agent.

The immune system functions in two distinct lines of defense: the innate immunity and the adaptive (or acquired) immunity. Types of immune response classified as “innate” are present already in almost all metazoans and depend on germ line-encoded receptors that recognize highly conserved pathogen-associated molecular patterns. These responses are extremely fast and constitute the earlier line of action of the immune system. In addition to innate immunity, jawed vertebrates have evolved an adaptive immune system that provides highly specific immune responses to particular pathogens. In contrast to the immediate induction of defense mechanisms from innate immunity, adaptive immune responses can take several days to mount. However, acquired immunity can confer in many cases lifelong protection to re-infection with the same pathogen, a phenomenon also known as immunological memory (Pancer and Cooper, 2006).

Innate immunity action is based on the recognition and killing of invading agents by phagocytes, a class of white blood cells (or leukocytes) specialized in the ingestion and digestion of unwanted materials. This cell type includes neutrophils and macrophages, which are the major players of the innate immunity. Other cells, called dendritic cells, are highly specialized in capturing antigen and present it to cells of the adaptive immune system. Cells of the innate immunity have a relatively short life-span and are thought to proliferate only in the generative hematopoietic tissues. Phagocytes express a large panel of surface receptors, such as integrins, scavenger receptors, Toll-like receptors, and others,

that recognize microbial conserved components and through which they can be activated to become effector cells.

If the innate immune defenses are by-passed, evaded or overwhelmed, an adaptive immune response normally ensues. Adaptive immunity is mediated by a very important class of leukocytes with potential for self-renewal and clonal expansion that carries on the surface specific recognition molecules, the antigen receptors. Such cells are called lymphocytes and fall into two major categories: B lymphocytes (or B cells) and T lymphocytes (or T cells). B lymphocytes are the major players of humoral immunity, and T cells are responsible for cell-mediated immunity, but often they work in concert, and also with other types of cells, to accomplish an effective immune response.

2. Lymphocyte ontogeny and diversity

All the cellular components of immune system develop from a population of multipotent and self-renewing hematopoietic progenitors, the hematopoietic stem cells (HSCs), which can be found in the adult bone marrow. HSCs differentiate into two main lineages: myeloid, which originates erythrocytes, megakaryocytes, granulocytes and monocytes, and lymphoid, which generates B, T and natural killer (NK) cells (Laiosa et al., 2006).

Adult mammalian B lymphocytes develop in the bone marrow in a sequence of discrete stages that can be identified by the differential expression of several surface markers and are associated to the progressive rearrangement of the immunoglobulin (Ig) loci. These events lead to the formation of a highly specific B cell receptor (BCR) which is expressed on the surface of mature B cells. Once activated, B lymphocytes differentiate into plasma cells, which are highly specialized in antibody production and secretion. The antibodies, which indeed correspond to a secreted form of the BCR, specifically bind the antigen that led to B cell activation. This can result in neutralization or opsonization that leads to pathogen destruction by phagocytes or complement.

T-cell precursors also originate from adult bone marrow-derived cells. Although it is not yet clearly established where T-cell commitment occurs, T-cell precursors eventually migrate at a very early stage to the thymus, where the receptor gene rearrangements and T-cell maturation can occur. Similarly to B lymphocytes, T-cell differentiation is a stepwise process towards the generation of T-cell receptor (TCR) expressing cells.

Unlike B cells, T lymphocytes recognize non-soluble antigens presented by highly polymorphic cell surface molecules generally known as major histocompatibility complex (MHC). T cells are said to be MHC restricted because they specifically recognize antigenic peptides presented by particular MHC alleles. MHC restriction is achieved during thymic T-cell development by positive selection of T lymphocytes recognizing self MHC-peptide complexes. In addition, a process of negative selection also takes place during T-cell maturation, leading to the deletion of lymphocytes with strong affinity to self peptides bound to self MHC molecules. Together, these two selection processes confer T lymphocytes the capacity of discrimination between “nonself” and “self”.

NK cells are also generated from the differentiation of common lymphoid progenitors. Unlike B and T lymphocytes, NK cells do not express clonally distributed receptors for antigen. For this reason, they are considered to belong to the innate immune system. However, they express a panoply of receptors, including inhibitory receptors specific for polymorphic MHC molecules. Such receptors enable NK cells to mediate “missing self recognition”, the capacity to attack self cells that extinguish expression of MHC class I molecules, such as viral infected or tumor cells. NK cells can thus recognize and exert strong cytolytic activity against cells with abnormal MHC expression, and also produce cytokines and chemokines that stimulate other immune functions.

2.1. A glance on T-cell development

The rearrangement status of TCR genes, together with the expression of TCR, the co-receptors CD4 and CD8 and other molecules on the cell surface, allow the discrimination of successive stages of T-cell differentiation in the thymus. Recombination events assemble the TCR coding sequence, which includes the variable (V), diversity (D), joining (J) and constant (C) gene segments that locate discontinuously in the genome. The program of T lymphocyte development can drive thymocytes into two distinct lines of differentiation: the γδ lineage, in which lymphocytes bear antigen receptors formed by a γδ heterodimer, and the αβ lineage in which the generated receptors consist of αβ heterodimers. For simplicity and because the aim of this manuscript does not cover the lymphocyte development issue, differentiation of γδ T-cells was omitted and only αβ T-cell development is described.

In brief, TCR β-chain DJ rearrangements occur first, followed by VDJ recombination in a stage where thymocytes are triple-negative for CD3, CD4 and CD8 and are $CD44^{low}$ $CD25^{+}$. A further productive rearrangement with a C gene segment allows the expression of low levels of the TCR β-chain at the surface, together with the invariant pre-α (pTα) molecule, a surrogate α-chain, and the CD3 complex. In this stage, $CD4^{-}CD8^{-}$ thymocytes have lost the expression of CD25. The expression of this pre-TCR signals the developing thymocyte to cease further rearrangements in the TCR-β locus (allelic exclusion) and to proliferate (β selection). After the proliferative burst, thymocytes acquire the expression of both

CD4 and CD8 co-receptors and rearrangements on the TCR α-chain locus begin. Rearrangements of the α-chain gene can occur continuously in both chromosomes even after the formation of a cell-surface receptor, which can generate in some cells more than one type of α-chain. These recombination events are ceased when $CD4^+$ $CD8^+$ double-positive cells undergo positive selection. This process favors the survival of thymocytes whose receptors recognize peptides bound to self MHC molecules and therefore occurs only after the expression at the cell surface of a productive αβ TCR. At this point, the type of co-receptor that will be expressed by mature T lymphocytes is also selected. Hence, TCRs recognizing peptides presented by MHC class II molecules lead to selection of the CD4 co-receptor, whereas lymphocytes whose TCRs recognize peptides presented by MHC class I molecules will develop into CD8-expressing cells. Positive selection therefore ensures that mature T-lymphocytes have antigen receptors capable of responding to peptides presented by self MHC molecules, selects the appropriate co-receptor and determines the functional commitment to the class of MHC molecule recognized. Finally, an additional process of negative selection leads to apoptosis of T cells bearing antigen receptors with high affinity to self peptides, thereby preventing the maturation of potentially self-reactive lymphocytes (Spits, 2002).

2.2. Diversity of T-cell repertoire

TCR diversification occurs primarily in the thymus by stochastic recombination of VDJ gene segments. Diversity is further increased by imprecise joining of the recombinant segments, by random addition of non-germline nucleotides by DNA-repair machinery and through pairing of diverse TCR α and β chains, or γ and δ chains. In addition, diversity can be still enhanced in cells expressing more than one type of TCR due to the lack of TCR α-chain allelic exclusion, which results in the pairing of more than one TCR α-chain with the same type of β-chain. Whether these “secondary” TCRs have an effective function with self MHC molecules is not clear. The diversity of αβ-TCRs collectively originated by these events was estimated to be higher than 1×10^{15}. Nevertheless, during intrathymic lymphocyte differentiation positive and negative selection significantly limit and shape the diversity of the generated repertoire by the loss or

enrichment of certain TCR specificities. The theoretical diversity of the peripheral TCR repertoire generated by this system was nevertheless estimated to be higher than 1×10^{13} – more than the number of T lymphocytes existing in a mouse ($1\text{-}2\times10^{8}$) or in humans (1×10^{12}) (Nikolich-Zugich et al., 2004). Moreover, some lymphocytes express the same TCR due to homeostatic proliferation of naïve T cells and/or homeostatic and antigen-driven expansion of effector and memory populations. The potential achievable repertoire is therefore several orders of magnitude larger than the one that can be expressed in an individual. This extra variability is probably the basis of the differences observed in the expressed TCR repertoire of genetically identical or different individuals.

Surprisingly, estimates of the real diversity of TCR repertoire in mice and humans indicate that it may be much lower than the number of T lymphocytes these organisms can comprise. In humans, the actual number of different $\alpha\beta$-TCRs was calculated to be about $2{,}5\times10^{7}$ and in mice 2×10^{6} (Nikolich-Zugich et al., 2004). If these estimates are correct, the restriction of diversity in humans is much more important as compared to mice, which can be due to the longer longevity of humans, the need of higher clone sizes in this species, the fact that mice are genetically manipulated animals that are maintained in protected environments, etc.

In addition to the previously described mechanisms that generate TCR diversification, the spectrum of specificities of a given TCR is still increased by crossreactivity with several ligands, as TCRs can vary in the degree of specificity/promiscuity of peptide-MHC recognition.

In summary, several mechanisms ensure the diversification of T-lymphocyte specificities. Because T-cell function relies on the ability to recognize the antigen presented by other cells; because the antigen universe is tremendously vast; and, finally, because the precise antigens derived from invading pathogens are unpredictable, the generation of a repertoire of T lymphocytes capable of responding to a broad range of antigens is fundamental for an efficient protection against pathogens.

3. T lymphocytes: key players on adaptive immunity

T lymphocytes are specialized in detecting and destroying pathogens that replicate inside the cells, such as some bacteria and other intracellular parasites, as well as viruses. Furthermore, T lymphocytes play a fundamental role in the immune surveillance against tumors.

T cells fall into two main classes depending on the type of co-receptor molecule expressed. Lymphocytes expressing CD8 specifically bind peptides presented by MHC class I molecules, which derive from proteins synthesized and degraded in the cytosol. In turn, $CD4^+$ T lymphocytes recognize peptides derived from proteins degraded in endocytic vesicles, which are presented by MHC class II molecules. These two types of T cells play distinct roles upon activation: $CD4^+$ T cells differentiate into cells capable of delivering different types of signals to other cells, such as B lymphocytes and macrophages, and $CD8^+$ T lymphocytes acquire the ability of killing other cells, i.e., become cytotoxic. As both $CD4^+$ and $CD8^+$ T cells recognize foreign peptides presented by the MHC proteins of the target cells, T-cell function relies mainly on direct interactions between T lymphocytes and the cells presenting the antigen. In addition, T lymphocytes can also product cytokines that may have stimulatory or inhibitory effects in different types of cells and contribute, directly or indirectly, to pathogen clearance.

3.1. Structure and function of the TCR

T cells bear on their surface thousands of antigen receptor molecules that recognize specific antigen determinants presented by MHC molecules (Figure 1). TCRs are made up of two membrane anchored polypeptide chains, either α and β in $\alpha\beta$ T cells or γ and δ in $\gamma\delta$ T cells (Garcia and Adams, 2005). Although they do not follow exactly the same differentiation processes, both $\alpha\beta$ T cells and $\gamma\delta$ T cells develop mostly in the thymus. In mice and humans, $\gamma\delta$ T cells represent 1-5% of circulating T lymphocytes, but in epithelial tissues most cells express $\gamma\delta$ receptors. The receptors of some of these epithelial $\gamma\delta$ T cells show very restricted variability. In contrast, $\gamma\delta$ T cells found in the blood, peripheral lymphoid organs or certain epithelial tissues as the gut, display highly diverse TCRs. Unlike $\alpha\beta$ T cells, lymphocytes bearing a $\gamma\delta$ TCR are not MHC restricted. They are implicated in

immunoregulation processes and tumor surveillance, as well as in some particular primary immune responses and wound healing. However, T cells that generally are responsible for antigen-specific cellular immunity are of the αβ type (Pennington et al., 2005). For this reason, this revision will focus only on αβ T cells and the generic terms TCR and T cell will always refer to the αβ T cells.

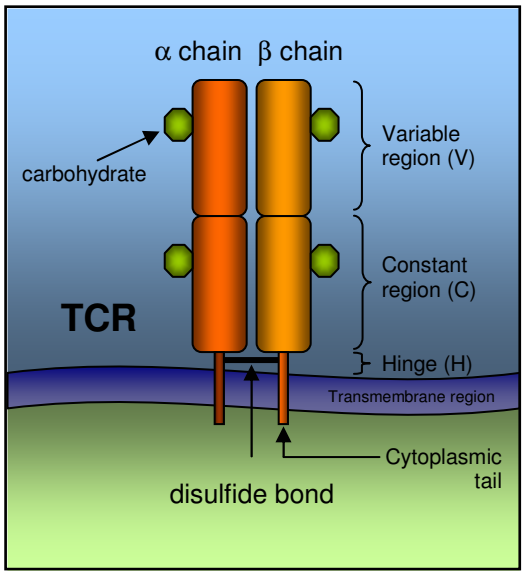

Figure 1. Structure of the T-cell receptor (adapted from Janeway et al., 1999)

During early T-cell development in the thymus, in a stage that precedes the rearrangement of the TCR α chain locus, thymocytes express a third type of TCR, in which the β chain pairs with a pTα chain. This pTα chain serves as surrogate for the TCR α chain and is actively involved in signaling via the pre-TCR (Call and Wucherpfennig, 2005).

Both α and β TCR polypeptides contain one constant and one variable regions. The antigen recognition site of the TCR is located in the variable domain and is formed by six loops named complementarity determining regions (CDRs). Each TCR chain contributes with three loops: CDR1, CDR2 and CDR3. The sequences for CDR1 and 2 are encoded by the V gene segments, but the outstanding TCR diversity relies on CDR3, which is created by the combinatorial and junctional processes between the V, D and J gene segments. In fact, the TCRα chain has a large number of J gene segments that, together with the diversity conferred by the D segments of the TCR β chain, accounts for the almost unlimited variability of the TCRs (Arden, 1998).

3.2. The CD3 complex

The functionally complete TCR comprises also the CD3 invariant chains, which associate non-covalently with the variable α and β chains of the TCR (Call and Wucherpfennig, 2005). The CD3 complex subunits include the γ, δ, ε and ζ chains, with all but CD3ζ being structurally related (Figure 2). Indeed, CD3γ, CD3δ

and CD3ε are encoded in adjacent genes, while ζ chains are encoded elsewhere in the genome. The CD3γ, CD3δ and CD3ε proteins contain an Ig-like extracellular domain, a transmembrane region and a cytoplasmic tail that contains one single immunoreceptor tyrosine-based activation motif (ITAM). In turn, CD3ζ is largely intracytoplasmic, having only a short extracellular portion, and own 3 ITAMs in its cytoplasmic domain.

Although the precise stoichiometry of the components that constitute a minimal TCR complex remains controversial, it is generally accepted that each αβ heterodimer associates with one CD3εγ heterodimer, one CD3εδ heterodimer and one CD3ζζ homodimer. In this way, each TCR would contain 10 ITAMs. Apparently the assemblage of TCR and CD3 chains depends largely on the interaction of the transmembrane domains, where the positively charged residues of the TCR interact with the negatively residues of the CD3 subunits (Arnett et al., 2004; Call and Wucherpfennig, 2005; Rudolph et al., 2006).

When the αβ heterodimer recognizes a specific peptide presented by the MHC complex, a cascade of signaling events are initiated via the ITAMs. The TCR αβ chains, however, have no functional relevant cytoplasmic domains and thus are not able to signal to the cell that antigen has bound. Therefore, while the αβ heterodimer recognizes and binds antigen, the CD3 complex is fundamental to transmit information from the external environment into the intracellular compartment, in form of signal transduction (Samelson, 2002).

The CD3 subunits are also required for normal surface expression of the TCR αβ heterodimer in mature T lymphocytes. The expression of TCR-CD3 complex is regulated in such a way that the absence of CD3 subunits results in defective or null TCR expression (Ashwell and Klusner, 1990). Moreover, deficiencies in the genes coding for the different CD3 subunits, which also associated with the pre-TCR, induce a blockade on thymocyte development before the $CD4^+CD8^+$ stage and impair the TCRα gene rearrangement. These evidences indicate that CD3 components have a role in the T cell differentiation (de Saint Basile et al., 2004; Fischer et al., 2005).

In summary, the TCR consists of two functionally distinct types of components: two genetically variable chains, which account for the recognition and binding to the antigen/MHC complex, and the invariant chains of the CD3 complex that interact with the intracellular proteins involved in signal transduction.

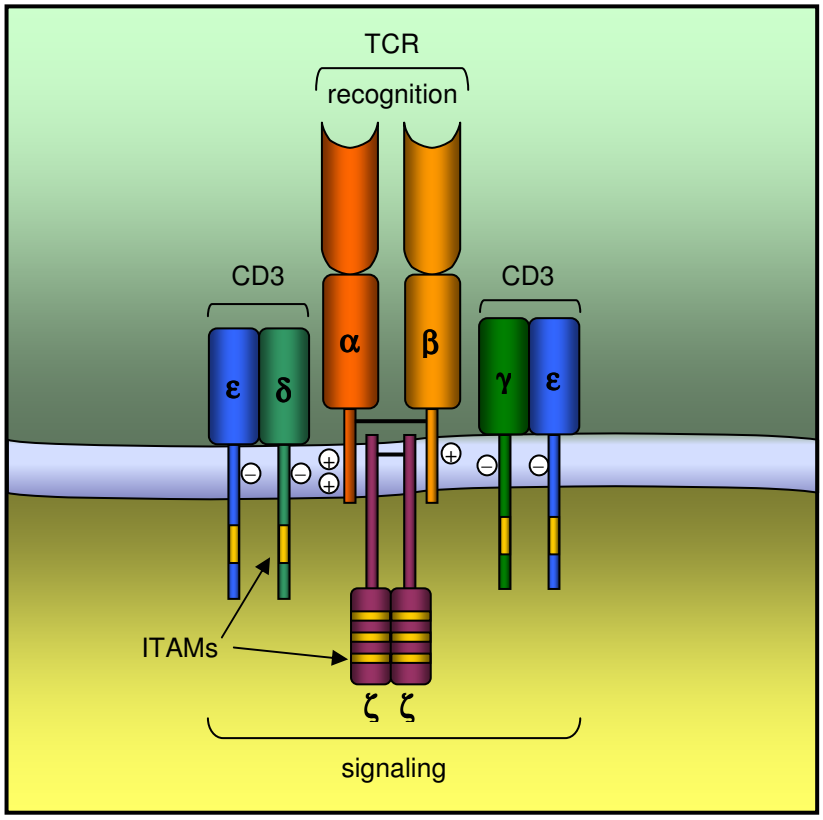

Figure 2. Simplified structure of the TCR-CD3 complex (adapted from Janeway et al., 1999).

3.3. The T cell co-receptors CD4 and CD8

T-cell co-receptors, CD4 and CD8, are cell surface glycoproteins expressed by distinct subsets of mature T lymphocytes (Figure 3). Hence, lymphocytes are referred to as $CD4^+$ or $CD8^+$, depending on the type of co-receptor they express.

During antigen recognition, the co-receptors associate with the TCR and bind the lateral face of MHC molecules. While CD4 associates with a constant domain of the MHC class II molecule, CD8 binds to invariant parts of the MHC class I molecule. Both co-receptors interact with MHC molecules in a domain that is distant from the TCR binding site, enabling the simultaneous association of CD4 and TCR, or CD8 and TCR, to the same peptide-MHC complex. Binding of CD4 or CD8 to the same peptide-MHC complex than the TCR is believed to be required for T cell activation and is on the basis of the classification of CD4 and CD8 as co-receptors (Janeway, 1992).

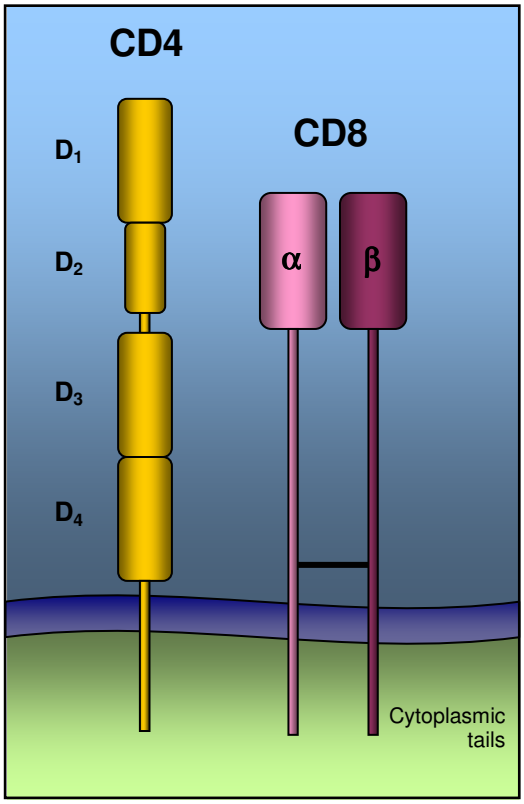

Figure 3. Structure of CD4 and CD8 molecules. CD4 is a single polypeptide chain molecule, made of four external immunoglobulin-related domains. Domains 1 and 2 (D1 and D2) have a unique strand topology, similarly to domains 3 and 4 (D3 and D4), which are joined to the first domains by a flexible hinge. The CD8 molecule is a disulfide-linked heterodimer of two polypeptides, α and β. Both chains contain an immunoglobulin-like amino-terminal domain linked to the membrane by an extended polypeptide region containing several O-linked sugars, which helps to maintain this stretch of polypeptide in an extended conformation and to protect it from cleavage by proteases. CD8α chains can also form homodimers, although these are not seen when the β chains are available (Zamoyska, 1998).

One of the major functions of CD4 and CD8 is to increase the avidity of the interaction between TCR and MHC complex (Hampl et al., 1997; Luescher et al., 1995). Furthermore, the cytoplasmic domains of CD4 and CD8 molecules interact strongly with components involved in the signaling cascade initiated upon antigen recognition, in particular the protein tyrosine kinase Lck (Veillette et al., 1988). Hence, the simultaneous binding of the co-receptors and the TCR to the same MHC:peptide complex brings Lck into close proximity to its targets, which associate with the cytoplasmic domains of the TCR complex. This enhances the tyrosine phosphorylation and further recruitment and activation of downstream signaling effector molecules (Germain, 2001).

In summary, mature T cells that recognize antigen in the context of MHC class I molecules express CD8, whereas T cells restricted by MHC class II express CD4. Aggregation of the appropriate co-receptor with the TCR enhances enormously the sensitivity of a T cell to the antigen presented by MHC molecules. In addition, optimal signaling trough the TCR occurs only when it clusters with the co-receptors CD4 or CD8.

3.3. T-cell receptor signaling

3.3.1. Signaling events initiated upon antigen recognition

The ITAMs are tyrosine-containing motifs that serve as sites of association with protein tyrosine kinases (PTKs) and other phosphotyrosine-binding moieties involved in receptor signaling. Upon antigen binding, the co-receptors CD4 or CD8 are clustered with the TCR complex. Consequently, the receptor-associated PTKs are brought together and act on each other and on the receptor cytoplasmic tails to initiate the signaling process.

The phosphorylation of the tyrosine residues of the ITAMs is the first intracellular signal indicating that specific antigen has been encountered. Two members of the Src family of PTKs mediate ITAM phosphorylation: Lck and Fyn. Lck interacts constitutively with the cytoplasmic domain of the co-receptor molecules CD4 and CD8 and is the predominant enzyme involved in ITAM phosphorylation (Rudd et al., 1989; Veillette et al., 1988). Fyn binds to the cytoplasmic domain of ζ and CD3ε chains upon receptor clustering. Both Fyn and Lck phosphorylate specific ITAMs on the accessory chains of the TCR complex (Samelson, 2002).

The activity of Src-family kinases (SFKs) is regulated by the phosphorylation status of two regulatory tyrosine residues, one activating and the other inhibitory, on the enzyme active site. In thymocytes and T cells both tyrosine residues are usually not phosphorylated. In this state, the SFKs are ready to be activated and are said to be in a “primed” state. During the receptor clustering that follows antigen recognition, the SFKs are brought in close proximity and transphosphorylation events can occur, leading to the activation of SFKs via their activating tyrosine. In contrast, phosphorylation of the inhibitory tyrosine induces SFK to adopt a closed conformation that renders the enzyme inactive (Palacios and Weiss, 2004). Lck and Fyn can, thus, be simultaneously present in the cell in three different states: in an open and non-activated conformation (primed); in an open and activated conformation (phosphorylated in the activating tyrosine); and in a closed and inactivated conformation (phosphorylated in the inhibitory tyrosine). These forms can exist in an equilibrium that might be shifted by CD4 or CD8 co-receptor ligation, or by the action of SFK regulatory enzymes, such as Csk or CD45.

3.3.1.1. The regulation of the Src-family kinases Lck and Fyn

Csk

The PTK Csk (C-terminal Src kinase) is a key element in controlling the activity of SFKs due to its ability to phosphorylate their inhibitory tyrosine. In resting cells, Csk constitutively associates to transmembrane proteins. Membrane anchoring favors Csk proximity to SFKs that, in this way, are maintained in an inactive state by phosphorylation of the inhibitory tyrosine. Upon TCR ligation, Csk is released from its membrane anchor into the cytosol, which likely contributes for the activation of Lck and Fyn (Palacios and Weiss, 2004).

CD45

The transmembrane tyrosine phosphatase CD45, also known as leukocyte common antigen, has the opposite effect of Csk. This phosphatase specifically dephosphorylates the inhibitory tyrosine residue in the C-terminus of SFKs and it is considered to be a critical positive regulator of Lck and Fyn in T cells (Hermiston et al., 2003). Hence, the balance between the action of Csk and CD45 is one of the ways in which the activity of SFKs is regulated.

All hematopoietic cells, with the exception of erythrocytes, express CD45 proteins. In lymphocytes, expression of CD45 is so abundant that can reach 10% of their surface area (Dahlke et al., 2004).

Structurally, the CD45 is a transmembrane protein with an extracellular region, a single transmembrane domain and a cytoplasmic tail. Only the cytoplasmic region, which contains two tandem tyrosine phosphatase domains, is required for enzymatic activity (Trowbridge and Thomas, 1994).

CD45 exists as distinct isoforms generated by alternative splicing of the exons 4, 5 and 6 that encode part of the N-terminal extracellular domain. At least eight isoforms can be theoretically generated that are distinguished on the basis of antibody recognition. In this way, the isoforms containing the product of exon 4, 5 or 6 are named CD45RA, CD45RB or CD45RC, respectively. The isoform generated by the complete splicing out of the three exons is called CD45R0. The amino-terminal region encoded by the exons 3 to 8 contains several O-linked

carbohydrates attachment sites. Therefore, the variable use of exons 4, 5 and 6 changes the size, shape and overall charge of the extracellular domain (Trowbridge and Thomas, 1994). Alternative splicing of CD45 occurs during T-cell development and peripheral activation. The pattern of isoform expression is highly conserved amongst vertebrates, suggesting functional importance *in vivo* (Okumura et al., 1996).

The natural ligand for CD45 has not yet been identified and the mechanisms involved in the regulation of CD45 activity are only beginning to be understood. The high regulation of isoform expression and the abundance of CD45 on the cell surface suggest a mechanism for regulating CD45 activity based on the spontaneous and isoform-differential homodimerization. Artificial dimerization of chimeric CD45 molecules inhibits the phosphatase function apparently by blocking the catalytic domain on the paired enzymes (Desai et al., 1993; Majeti et al., 1998). Disruption of this regulatory mechanism leads to autoimmunity and lymphoproliferation in mice (Majeti et al., 2000). The extracellular domains of the different CD45 isoforms, which exhibit different numbers of O-glycosylation modifications, have a major influence in the homodimerization and, consequently, the activation status of the protein (Xu and Weiss, 2002). For instance, smaller molecular isoforms, which are less O-glycosylated, dimerize more efficiently than larger isoforms.

In human cord blood almost all $CD8^+$ T cells express the high molecular weight (m.w.) isoform CD45RA (Hamann et al., 1997). With increasing age, $CD8^+$ T cells express also the CD45R0 isoform. Actually, T cell activation induces a shift from high to low CD45 m.w. isoforms (Akbar et al., 1988). Consequently, the expression of CD45RA is down-regulated while CD45R0 expression is up-regulated. In addition, expression of CD45RA appears to be re-acquired later in the immune response, with the concomitant loss of CD45R0 (Champagne et al., 2001). These changes in cell-surface phenotype are one of the ways to distinguish naïve from antigen experienced cells, effector and memory, and will be discussed in more detail later in this manuscript.

3.3.2. TCR signaling final destination: the nucleus

TCR activation following antigen recognition leads to phosphorylation of ITAMs. In the phosphorylated state, ITAMs can recruit the zeta-associated kinase (ZAP-70), which binds to the ITAMs in the ζ chain via its tandem SH2 (Src homology domain 2) domains. Recruitment of ZAP-70 allows its phosphorylation and enzymatic activation by either Lck or Fyn. Active ZAP-70 then phosphorylates the membrane adapter protein LAT (linker of activation in T cells). LAT contains multiple tyrosines that, once phosphorylated, serve as SH2-domain-binding regions. The cytoplasmic adapter SLP-76 (SH2 domain containing leukocyte phosphoprotein of 76 kDa) binds to phosphorylated LAT, and together these proteins act as sites for the recruitment of additional adapters and key enzymes involved in further downstream signaling events in T cells (Germain, 2001).

Two main types of proteins containing SH2 domains participate in the signal cascade initiated by TCR triggering. The first one is phospholipase C-γ (PLC-γ), which initiates two of the main signaling pathways leading to the nucleus. The second is Ras, a small G protein that initiates the third and major pathway, which consists in a cascade of protein kinases that leads directly to the phosphorylation and activation of transcription factors.

PLC-γ pathways

PLC-γ is recruited to the cell membrane by its SH2 domains, where it is activated by phosphorylation of a tyrosine residue. In the active state, PLC-γ cleaves a molecule of the phospholipid membrane, the phosphatidylinositol bisphosphate (PIP_2), into inositol trisphosphate (IP_3) and diacylglycerol (DAG, Figure 4). One single molecule of PLC-γ is able to generate many molecules of DAG and IP_3. Therefore, this and similar enzymatic steps contribute importantly to amplify and sustain the signal initiated by TCR ligation. Noteworthy, production of DAG and IP_3 by activated PLC-γ is not exclusive from the TCR-signaling cascade, being rather a common step in signaling pathways of several types of receptors.

After cleavage of PIP_2, diffusion of IP_3 induces the release of Ca^{2+} from intracellular storage sites in the endoplasmic reticulum (ER) into the cytosol. Intracellular free Ca^{2+} levels increase drastically, triggering the opening of calcium channels in the plasma membrane, which let in more Ca^{2+} into the cell, thus sustaining the signal. High levels of free Ca^{2+} induce activation of the Ca^{2+}-binding protein calmodulin, which can associate to and regulate the activity of several other proteins, propagating the signal onwards along pathways that also eventually converge to the nucleus.

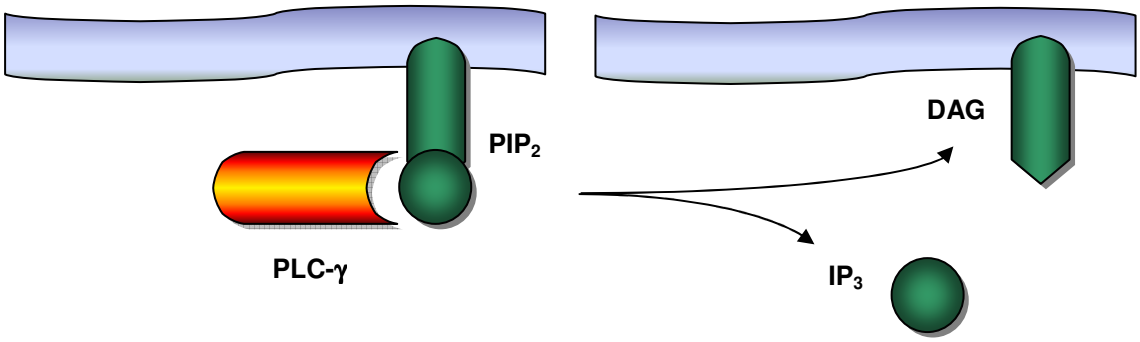

Figure 4. Cleavage of PIP_2 into DAG and IP3, an intermediate step in the signaling cascade initiated upon TCR stimulation.

The other product of PIP_2 cleavage, DAG, remains linked to the plasma membrane and contributes to the activation of the protein kinase C (PKC). This serine/threonine kinase is believed to be important in initiating one of the main signaling pathways leading to the nucleus. PKC is additionally activated by the raise of Ca^{2+} concentration due to IP_3 action. In this way, both products of the cleavage of PIP_2 reinforce each other in activating PKC (Janeway, 1999).

Ras pathway

Like the other small G proteins, Ras can be present in the cell in two different forms, depending on whether it is binding GTP or GDP. When bound to GTP, Ras is in the active state. Since this form has an intrinsic GTPase activity, Ras can turn itself off by removing a phosphate group of GTP. The GDP-bound form of Ras is therefore inactive and is the predominant form found in the cell.

Activation of small G proteins requires a guanine-nucleotide exchange factor (GEF), which exchanges GDP to GTP. In lymphocytes, Ras is recruited to

the antigen receptor complex by adaptor proteins to which GEFs also bind, making therefore possible the activation of Ras.

Activated small G proteins trigger a cascade of protein kinases named mitogen-activated protein kinases (MAP kinases). Localization and activity of MAP kinases depends on their phosphorylation status: non-phosphorylated MAP kinases are inactive and stay in the cytoplasm, but once phosphorylated become active and translocate into the nucleus.

MAP kinases pathway has three major enzymatic levels. The first enzyme in the cascade is a serine/threonine kinase, generally called MAP kinase kinase kinase (MAPKKK or MAPK3), which is activated by the GTP-bound form of Ras. In the active form, MAPKKK phosphorylates the next downstream enzyme, the MAP kinase kinase (MAPKK or MAPK2). This class of proteins has the ability to phosphorylate MAP kinases, the last level enzymes, on both a tyrosine and a threonine residue, which induces MAP kinase activation.

MAP kinases directly phosphorylate and activate transcription factors, a class of proteins that bind specific sites on DNA and regulate gene transcription. Transcription factors activated through MAP kinase pathway in consequence of antigen recognition usually regulate the expression of genes involved in proliferation, cell death and survival, inflammation and DNA repair (Dong et al., 2002).

Part II. T-cell immune responses

The most remarkable feature of the acquired immune system is the capacity to generate immunological memory, i.e., a faster, stronger and more effective response against an antigen that has already been cleared in a prior immune response.

In the course of a primary immune response, antigen presented by professional antigen-presenting cells (APCs) will drive specific naïve T-cell to proliferate and further differentiate. Several different outcomes are possible. In the case of $CD4^+$ T lymphocytes, differentiation will induce the polarization of lymphocytes toward a T helper 1 (T_H1) or T_H2 phenotype, originating cells highly specialized in cytokine secretion (INFγ or IL-4, respectively). T_H1 $CD4^+$ T lymphocytes are the main macrophage activators, potentiating inflammatory immune responses, whereas T_H2 $CD4^+$ T cells activate B lymphocytes and promote immune responses predominantly mediated by antibodies. In the other hand, $CD8^+$ T lymphocytes differentiation leads to the formation of cytotoxic T cells (CTL) that kill infected target cells. Upon differentiation T lymphocytes can also acquire regulatory properties, which enable them to modulate immune responses. In any case, cells generated upon differentiation are ready to respond quickly and efficiently upon encounter with the specific antigen and, thus, can be considered as “effector cells”.

In the sequence of naïve priming and differentiation, another type of cells can further be generated, which have less stringent requirements for activation than naïve cells, are able to proliferate and acquire effector functions in a reduced lag-time, and have increased survival capacity. As these cells can survive for years in the organism, they were termed “memory cells”.

1. Primary immune responses

1.1. APCs interact with naïve T cells in the secondary lymphoid organs

The effective onset of adaptive immune responses requires that naïve antigen-specific lymphocytes, being inherently rare throughout the body, rapidly encounter foreign antigens. This problem has been elegantly solved in evolution through the development of secondary lymphoid tissues as intersections in the migratory pathways of antigen-presenting dendritic cells (DCs) and antigen-specific lymphocytes. T-cell immune responses are therefore initiated in secondary lymphoid organs, where peptide-loaded APCs can encounter T cells bearing a specific TCR for the antigens they have uptaken in the periphery. Pathogens infecting peripheral tissues are captured by DCs that will be trapped in the lymph nodes directly downstream the site of infection. Nonetheless, antigen presentation can take place in other sites, such as the spleen, if infection takes place in the blood, or Peyer's patches and tonsils, when pathogens invade mucosal surfaces.

The secondary lymphoid organs are highly organized structures where B, T and antigen presenting cells can interact and initiate immune responses. Naïve T lymphocytes are continuously circulating from one lymphoid organ to another, via blood and lymph, until they encounter antigen. While migrating and prior to contact with specific antigen, naïve T cells are metabolically quiescent and have a prolonged lifespan, which depends on the successive contact with self-peptide/MHC complexes and a cytokine, interleukin-7. Recognition of these ligands presumably delivers low-level signals, which keep T cells sufficiently metabolically active to avoid passive death (Sprent and Surh, 2002; Tanchot et al., 1997).

The migratory properties of naïve T cells do not enable them to enter the peripheral tissues. Therefore, naïve T lymphocytes can only recognize specific antigen and initiate immune responses in the secondary lymphoid organs (Sprent and Surh, 2002) (Figure 5).

To get in lymph nodes and Peyer's patches, but not in the spleen, T cells must cross the walls of the high endothelial venules (HEV). This event requires the interaction of molecules present in the plasma membrane of lymphocytes, such as selectins and integrins, and their corresponding ligands expressed by the cells of the vascular endothelium. Of particular interest are the L-selectin (also known as

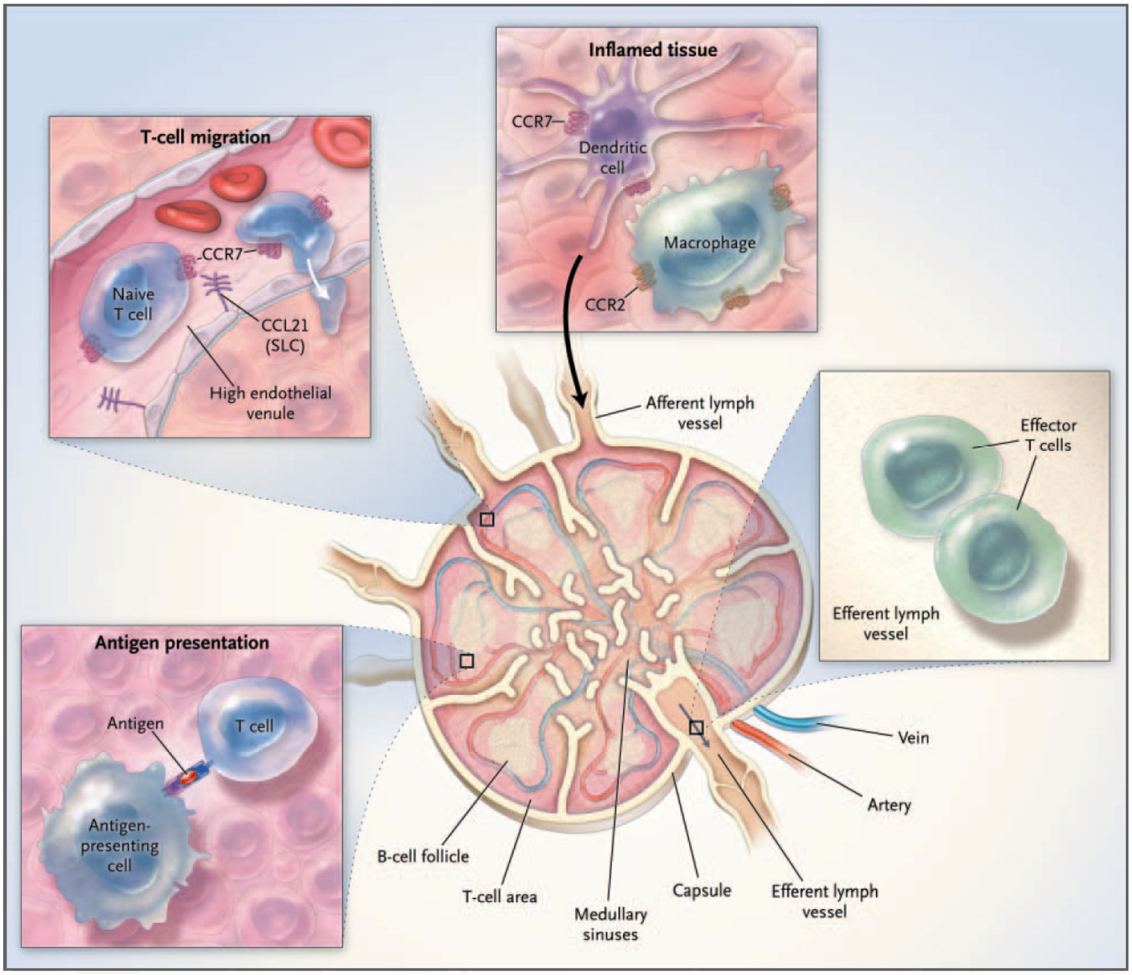

Figure 5. Trafficking of cells of the immune system through the lymph node.
Adapted from Charo et al., 2006.

CD62L) and the chemokine receptor CCR7 expressed on the surface of T cells. Interaction of L-selectin and the vascular addressins mediates T-cell rolling on the endothelium, which enables the interaction between CCR7 and its ligands, SLC and ELC (standing for, respectively, secondary lymphoid-organ chemokine, also known as CCL21, or Epstein–Barr virus-induced molecule 1 ligand chemokine, also known as CCL19). SLC and ELC are also expressed by stromal cells within the T cell areas in the lymphoid tissue and thus target T cells into these sites (Cyster, 1999; Zlotnik et al., 1999). Mice lacking CCR7 (through targeted gene deletion) or that have insufficient CCL19 or CCL21 (through naturally occurring mutation) have structurally disorganized lymph-node T-cell zones and are deficient in T-cell dependent immunity (Muller et al., 2003a). Interaction with ELC or SLC differs fundamentally in the ability to induce the internalization of CCR7, which occurs when this receptor interacts with ELC, but not SLC. Based on this finding, it was proposed that T lymphocytes enter lymphoid tissues mainly in response to

SLC produced by HEVs. In this way, lymphocytes would retain full chemotatic responsiveness to ELC and SLC produced at similar levels in T-cell zones, therefore maintaining the ability to migrate into those areas (Bardi et al., 2001).

When T lymphocytes reach the T cell-zones on the secondary lymphoid tissues they establish contact with APCs, in particular DCs, and scrutinize the peptides presented by MHC class I and II molecules on its surface.

Typically, DCs are dispersed in nonlymphoid tissues as resident cells in a resting, immature state. Immature DCs are very efficient in capturing antigen, but are not capable of efficiently present antigens and activate T cells. This capacity only emerges after a complex developmental process called “DC maturation”. The antigen uptake associated to other signals, like the cytokine and chemokine microenvironment generated by local inflammation, drives immature DCs to undergo phenotypic and functional changes that culminate in the complete transition from an “antigen-capturing” cell to an “antigen-presenting” cell specialization. These modifications include changes in morphology, such as formation of dendritic projections, upregulation of co-stimulatory receptors (like B7-1, B7-2, CD40 and CD58) and an overall increase in the levels of MHC class I and class II molecules on the surface. Another important property that accompanies maturation is the acquisition of high cellular motility and up-regulation of chemokine receptors, such as CCR7 (Mantovani, 1999). After antigen uptake DCs leave the inflamed tissues, enter the lymph stream and migrate to the secondary lymphoid organs. Here they are driven by gradients of CCL19 and CCL21 to the T-cell zones where contact with T cells takes place and the DC maturation process is completed (Trombetta and Mellman, 2005).

Interaction with T cells should start soon after DCs reach the T-cell zone because DCs are relatively short-lived cells, especially after activation, with a half-life as low as 1–2 days upon arrival in the lymph node (Ingulli et al., 1997; Kamath et al., 2002). Yet recent *in situ* imaging studies suggest that individual T–DC couplings may last 37 h or longer (Stoll et al., 2002). Longevity and abundance of DCs in the site of T-cell priming influence the capacity to prime and the strength of the activating signals delivered to T cells (Josien et al., 2000; Wong et al., 1997). Evidence that enhanced DC survival potentiates T-cell activation has been demonstrated by studies of TRANCE, a TNF family member expressed on T cells, which can trigger its receptor, also known as RANK, on DCs enhancing their

viability (Anderson et al., 1997; Wong et al., 1997). A role for CD40L in regulation of DC survival has also been demonstrated (Quezada et al., 2004).

The naïve T lymphocytes that do not encounter specific antigen leave the secondary lymphoid tissue by the efferent lymphatics and proceed circulation in the bloodstream and through the other secondary lymphoid organs. In contrast, if a specific peptide has been presented by the APC, T cells interrupt circulation to initiate a sequence of events that leads to the generation of effector cells. This can take several days and encompass two distinct processes: proliferation and differentiation. In the end, T cells can leave the lymphoid tissue also by the efferent lymphatics and re-enter the circulation to migrate to the sites of infection or, instead, stay within the lymph node to help B lymphocytes.

1.2. Requirements for T-cell activation

T-cell activation is a complex, multistep process involving a multitude of molecules. When T lymphocytes recognize the specific peptide on the surface of an APC, several protein-protein interactions are elicited in addition to the specific interaction between the TCR and the MHC-peptide complexes. As mentioned above, the co-receptors CD4 or CD8 are recruited to their binding sites on MHC class II or MHC class I, respectively, thereby lowering the threshold for T-cell activation. Moreover, interaction between co-stimulatory receptors, the most important of which is CD28, and their cognate ligands on the APC also occur. These co-stimulatory interactions are fundamental for effective lymphocyte activation and also enhance the immune response (Lenschow et al., 1996). Finally, it should be stressed out that for all these interactions to be possible, a stable adhesive junction must be formed between the T cell and the APC. Adhesion molecules like CD2, ICAM-3 or LFA-1, present on lymphocytes plasma membrane, play an important role in the efficient binding to APCs by interaction with molecules on their surface.

After antigen recognition, the avidity of LFA-1 for ICAM-1 (standing for intercellular adhesion molecule-1), an integrin expressed on the surface of the APC, increases rapidly (Dustin and Springer, 1989). This leads to the formation of an adhesion complex between the T cell and the APC characterized by a specific pattern of receptor segregation with a central cluster of TCRs and one of its

downstream signaling effectors, protein kinase C-θ (PKC-θ), surrounded by a ring of integrin family adhesion molecules. This complex is known as immunological synapse (Grakoui et al., 1999) or supramolecular activation cluster (SMAC) (Monks et al., 1998), and facilitates and sustains TCR engagement and signaling (Bachmann et al., 1997). Large molecules such as CD45, also interact with the APC but are excluded from the SMAC (Huppa and Davis, 2003).

In addition to the signal generated by TCR engagement, a second signal is often required to achieve full activation of T lymphocytes. This second signal is provided by ligation of molecules displayed on the lymphocyte surface and is complementary to TCR activation. For this reason, it is called co-stimulation and the molecules involved are known as co-stimulatory receptors.

The immune system has evolved in such a way that only APCs display the molecules capable of delivering co-stimulatory signals to lymphocytes. This is particularly important to avoid activation of self-reactive T cells that have escaped thymic negative selection and might recognize specific peptides displayed by MHC class I molecules of tissue cells. Hence, the need for co-stimulation constitutes also a strategy of discrimination between self and non-self. Indeed, lymphocytes receiving a TCR-mediated signal 1 in the absence of an additional, accessory molecule-mediated signal 2 become functionally tolerant (for a review, see Baxter and Hodgkin, 2002). This is generally known as the “two-signal theory” of lymphocyte activation and supports that signal 2 is important not only for complete T cell activation but also to prevent induction of unresponsiveness.

The major co-stimulatory pathway involves the binding of CD28 to its ligands, CD80 or CD86 (also known as B7-1 or B7-2, respectively). Co-stimulation by anti-CD28 antibody enhances T cell activation by decreasing the time of commitment, amplifying TCR signaling and protecting T cells from death (Iezzi et al., 1998; Lenschow et al., 1996; Viola et al., 1999). In addition to CD28, other molecules expressed by lymphocytes can mediate co-stimulation, such as several members of the tumor necrosis factor receptor (TNFR) family, like CD27 (Watts, 2005). The biology and function of CD27 and CD28, as well as their role in lymphocyte co-stimulation, will be discussed in further detail in the following section.

1.3. Co-stimulation may be also required for full activation of T cells

Optimal T cell activation is believed to require two signals: ligation of TCR to MHC-peptide complex (signal 1) and co-stimulation (signal 2). Several reports have described activation of CD8 cells without the need for co-stimulation, but this requires high dose or persistent antigen exposure and the use of TCR transgenic mice (Kundig et al., 1996; Luxembourg et al., 1998; Manning et al., 1997; Wang et al., 2000). In contrast, only in the presence of co-stimulation can low concentrations of antigen induce high CD69 expression, proliferation, and high IL-2 production. A critical role for co-stimulation in activation of $CD8^+$ CTL was also reported (Gajewski et al., 1996; Krummel et al., 1999).

Co-stimulation enhances T-cell activation in two distinct ways: by promoting a more efficient engagement of TCR or, alternatively, by providing additional signals to induce cell division, augment cell survival or trigger effector functions, such as cytokine secretion or cytotoxicity. Multiple membrane receptors were identified as being co-stimulatory. These include receptors belonging to the immunoglobulin superfamily, such as CD28, or members of the TNFR family, such as CD27. Signaling through these receptors can engage molecules common to the TCR signaling cascade, thus improving quantitatively the signal initiated by the recognition of specific antigen. Alternatively, co-stimulation can involve the activation of signaling intermediate molecules distinct from those engaged by TCR, which can selectively induce particular cellular mechanisms. Importantly, expression of co-stimulatory receptors by lymphocytes varies along the T-cell immune response and also in consequence of the cytokine environment. This probably reflects the need of distinct signals at different stages of the immune response, the spectrum of co-receptors defining the type of alternative functions that might be promoted (reviewed by Acuto and Michel, 2003; Croft, 2003).

1.3.1 CD28

CD28 is an integral membrane protein homodimer that contains in the extracellular region one immunoglobulin-like domain. In humans, CD28 is expressed by ~90% of $CD4^+$ and by ~50% of $CD8^+$ T cells (Acuto and Michel, 2003). CD28 binds to B7-1 (CD80) and B7-2 (CD86), two structurally homologous proteins, expressed on activated APCs, such as DCs, macrophages and activated

B cells. B7-1 and B7-2 also belong to the immunoglobulin superfamily and might exist as dimers and monomers, respectively (Figure 6). Mice deficient for either CD80 or CD86 show that they have partially overlapping functions (McAdam et al., 2000). Differences in expression kinetics between CD80 and CD86 might explain the functional differences that have been observed. CD86 expression is induced on APCs early in the immune response and, thus, might be required for initiating immune responses. CD80, in contrast, is expressed later and is thought to deliver to T cells a more powerful signal, which might be crucial for regulating immune responses, perhaps by interacting preferentially with the inhibitory CD28 analogue, cytotoxic T lymphocyte antigen 4 (CTLA4) (Acuto and Michel, 2003; Margulies, 2003).

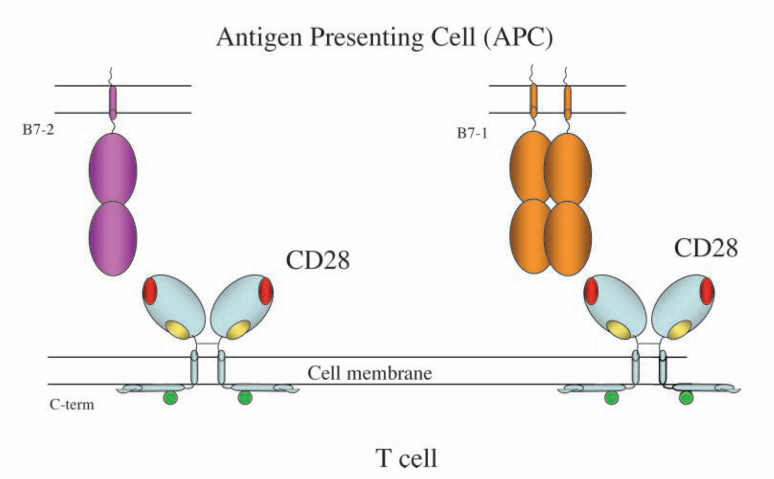

Figure 6. Schematic structure of CD28 and B7-1 and B7-2. Adapted from Margulies et al., 2003.

The main contribution of CD28-mediated signaling is to enhance IL-2 production (Lucas et al., 1995; Reichert et al., 2001) and IL-2Rα expression (McAdam et al., 1998; Shahinian et al., 1993), and accelerate entry into and progression through the cell cycle (Bonnevier and Mueller, 2002). However, IL-2, as well as other common cytokine receptor γ-chain-dependent cytokines, do not have an important role in antigen-driven T-cell proliferation *in vivo* (Kundig et al., 1993; Lantz et al., 2000) and *in vitro*, CD28-enhanced cell-cycle progression is partly IL-2 independent (Appleman et al., 2000).

Co-stimulation via CD28 was shown to be required for $CD8^+$ T cell activation in vesicular stomatitis virus and vaccinia virus infections, as well as in infection with *Listeria monocytogenes* (McAdam et al., 2000; Mittrucker et al., 2001). However, CD28 co-stimulation is assumed to not be necessary for CD8 T-cell priming following lymphocytic choriomeningitis virus (LCMV) infection (Suresh et al., 2001). Therefore, the role of CD28 co-stimulation in protective immunity

differs for different pathogens, probably reflecting disparities in the activation of innate inflammatory responses. Alternatively, the lack of co-stimulation might be compensated by a very strong signal delivered trough the TCR, which probably relates to the abundance of specific T cells and/or the affinity of the TCR to particular epitopes, or by an additional helper effect. The non-cytolytic nature of LCMV, for instance, might allow the delivery of a uniquely strong and prolonged signal 1. In addition, a recent study has reported that CD28 co-stimulatory signals might be fundamental for CTL priming in the absence of $CD4^+$ T-cell help (Voigt et al., 2006).

Ablation of CD28 signaling by gene knockout of the receptor or both ligands, or by treatment with antagonists of CD28 has several distinct consequences, including reduced T-cell proliferation *in vitro* and *in vivo*, severe inhibition of germinal-centre formation and immunoglobulin isotype-class switching, reduced T helper-cell differentiation, induction of T_H2-type cytokine expression and compromised cytotoxic $CD8^+$ T-cell responses (for a revision see Acuto and Michel, 2003). Since CD28 stimulation occurs early in the immune response by APCs that are probably stimulating the TCR in parallel, in situations of particular low TCR occupancy, CD28 is unique in providing a potent synergistic signal to naive cells to effectively activate transcription factors such as nuclear NF-κB, NFAT and AP1, which control cell proliferation, death and differentiation (Acuto and Michel, 2003). In addition, co-stimulation through CD28 up-regulates the expression of cytokines, including, IL-2, interferon-γ and IL-4 (McAdam et al., 1998); chemokines, e.g., macrophage inflammatory protein-1α (Herold et al., 1997); receptors for cytokines and chemokines, e.g., the IL-2 receptor, IL-12 receptor and CXC chemokine receptor 5 (CXCR5) (Park et al., 2001; Shahinian et al., 1993; Walker et al., 2000); and receptors such as CD40L, CTLA-4 or inducible co-stimulatory molecule (ICOS), that will bind their ligands on APCs (Sharpe and Freeman, 2002).These events ensure the delivery of a second wave of signals (hours or days after the initial T-cell-APC encounter) in secondary lymphoid organs and/or after the migration to tissues, and are crucial for effector T-cell functions and the establishment of long-term memory (Acuto and Michel, 2003).

1.3.2. CD27

CD27 is a protein evolutionarily conserved between mice and man that is generally expressed in homodimers of 55 kDa on lymphocytes membrane (Camerini et al., 1991; Gravestein et al., 1993; van Lier et al., 1987). A soluble form of CD27 (sCD27), likely generated by proteolytic cleavage of the surface molecule, can also be found in activated T lymphocytes (Borst et al., 1989; Hintzen et al., 1991a; Loenen et al., 1992).

CD27 has a constitutive expression on NK cells, antigen experienced B cells and naïve CD4 and CD8 T cells (reviewed by Croft, 2003; Watts, 2005). Similarly to CD28, expression of CD27 varies along the T-cell immune response and between distinct T-cell subsets (discussed later).

CD70, also known as CD27 ligand (CD27L), is a TNF family member and the natural ligand of CD27 (Bowman et al., 1994; Goodwin et al., 1993; Hintzen et al., 1994). In contrast to CD27, which is constitutively expressed on resting T cells, NK cells and memory B cells, CD70 expression is only transiently induced upon activation of T cells, B cells and NK cells and on mature DCs (Bertram et al., 2004; Kashii et al., 1999; Tesselaar et al., 1997; Tesselaar et al., 2003b; Watts, 2005). The expression patterns of CD27 and CD70 in humans and mice are quite similar, with the exception of a higher frequency of primed B cells expressing CD27 in humans (Borst et al., 2005).

CD70 expression is primarily controlled by antigen receptor and Toll-like receptor stimulation, but it can also be induced by anti-CD40 and GM-CSF. Following influenza viral infection, CD70 can be detected on T and B cells in draining lymph nodes (LNs) of mice. It can also be found in lung infiltrating T cells, but most of the protein is retained intracellularly, suggesting that CD70 expression at the surface is tightly regulated (Tesselaar et al., 2003b). In fact, CD70 over-expression leads to a lethal immunodeficiency attributed to chronic stimulation, which may explain the need of a tight physiological regulation of this molecule (Tesselaar et al., 2003a).

Interaction between CD27 and CD70 on T cells enhances TCR-induced expansion and promotes the generation of effector T cells with cytolytic function and IFN-γ-secreting capacity, as discussed below. The expression pattern of CD27 and CD70 suggest several different landscapes of interaction: (i) in the T-cell priming phase, during the interaction between T cells and DCs; (ii) in the

expansion phase, through T-T cell interactions; and (iii) in the effector phase, between CD27 expressed on T cells and CD70 on DCs and B cells, which express high levels of CD70 at the effector site (Borst et al., 2005 and Figure 7).

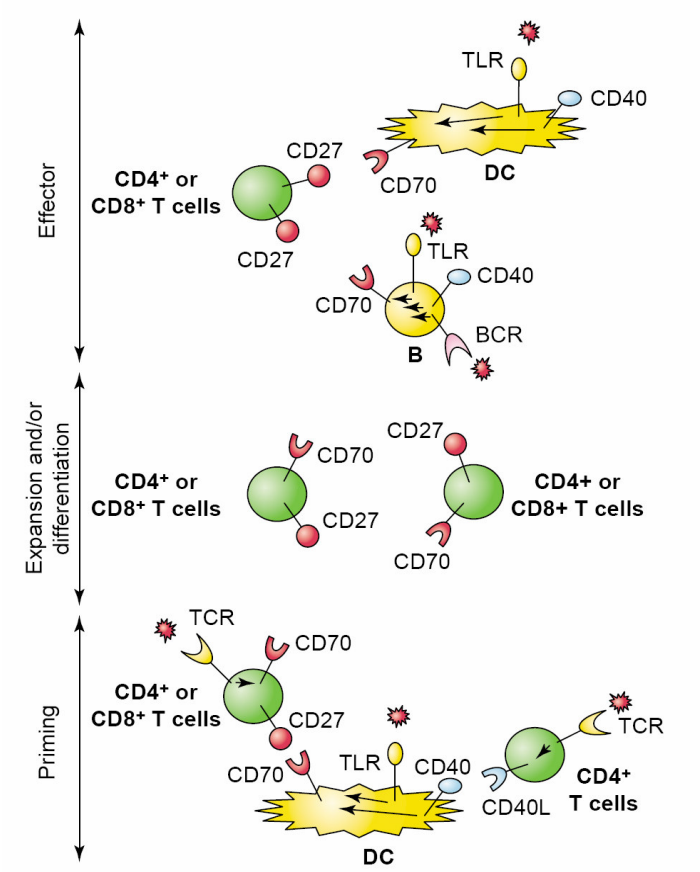

Figure 7. CD27-CD70 interactions in different phases of the immune response. Adapted from Borst et al., 2005.

Considering that co-stimulation refers to the need of a second signal in addition to those received through the TCR to achieve full T-cell activation and survival, CD27 can be considered a co-stimulatory molecule as its engagement enhances proliferation of $CD4^+$ and $CD8^+$ T cells, as well as cytokine production and survival. *In vitro* studies revealed that unlike CD28, stimulation via CD27 does not induce strong production of IL-2 by T-cells in murine cells. However, it elicits TNF-α production at comparable levels to CD28, promotes development of cytotoxic T lymphocyte (CTL) effectors and enhances T-cell survival (Brown et al., 1995; Hintzen et al., 1995; Hendriks et al., 2000; Ochsenbein et al., 2004).

Transgenic mice constitutively expressing CD70 show at 4 weeks of age increased numbers of $CD4^+$ and $CD8^+$ T cells displaying an effector phenotype. Nonetheless, these mice present a reduced number of mature B-cells due to a high CD27-dependent IFN-γ production by T-cells (Arens et al., 2001). The increment on IFN-γ production in CD70-Tg mice is not a mere consequence of a higher number of effector cells, but it is also due to increased IFN-γ production on a per cell basis. Consequently, mice over-expressing CD70 have enhanced CD8 T-cell responses to influenza virus, as well as improved tumor rejection (Arens et al., 2004). On the other hand, such a constant delivery of co-stimulation signals seems to exhaust the immune system, as these mice die between 6-8 months of age from *Pneumocystis carinii* infection, a hallmark of T-cell immunodeficiency (Tesselaar et al., 2003a). This lethal immunodeficiency is ascribed to an antigen-dependent conversion of the naïve T-cell pool into effector cells over time, thereby leading to the depletion of the naïve T-cell repertoire. This finding is in line with the tight regulation of CD70 expression observed *in vivo*. In fact, analysis of lung tissue and LN-derived cells of mice infected with influenza showed that CD70 expression can be found in lung-infiltrating T cells, but not on B cells or $CD11c^+$ DCs. Furthermore, it can be expressed on B and T cells recovered from LNs as well. In contrast, CD70 expression was virtually absent from lymphocytes of the LNs and lung of uninfected mice. Interestingly, confocal microscopy revealed that CD70 expression on the LN-derived cells was largely cytoplasmic, particularly on B cells. In addition, flow cytometry studies showed that plasma membrane expression of CD70 was detected only in a small fraction of $CD3^+$ T-cells and this fraction was maximal on the peak of the response. These data indicate that CD70 is induced in T and B cells upon *in vivo* activation, but is to a large extent retained intracellularly by transcriptional and post-translational mechanisms not disclosed yet (Tesselaar et al., 2003b).

In CD27-deficient mice, reduced numbers of $CD4^+$ and $CD8^+$ effector T cells were found in the lung after primary and secondary infection with influenza virus (Hendriks et al., 2000). These observations imply that CD27/CD70 interaction regulates the size of the expanded T-cell pool *in vivo*. However, CD27 does not affect cell division of T cells, as no difference is found between the carboxyfluorescein diacetate succinimidyl ester (CFSE) profiles of wild-type and CD27 KO cells. Unlike CD28, that increases IL-2 production thus enhancing entry in cell cycle, CD27 rather appears to promote T-cell expansion by allowing

activated T cells to survive throughout successive rounds of division, especially at the site of infection. This is achieved by promoting the generation of a specific T-cell pool on the LNs and, possibly, by the induction of lymphocyte migration to the site of infection and/or their survival at this site (Hendriks et al., 2003).

The relative contributions of CD27 and CD28 co-stimulatory signals for cellular accumulation depend on the organ and on the type of response. Hence, during primary response to influenza virus in mice, generation of virus-specific CD8 T-cell pool in the draining LNs is highly dependent on the collective, partially non-redundant contribution of both CD27 and CD28. In the lungs, however, the absence of CD28 can be greatly compensated by CD27 as T-cell response at this site remains partially intact if CD27 signaling is available. Conversely, the secondary response in the lungs is more dependent on CD28 than primary response, while in LNs it remains relatively similar. In the spleen, the response is regulated in a slightly different way. In the primary response, lack of CD28, but not CD27, impairs at a great extent the accumulation of virus specific CD8 T cells, whereas upon secondary challenge memory responses were severely reduced by either CD27 or CD28 depletion. This evidence indicates that the role of CD27 in the spleen is much more significant in secondary responses (Hendriks et al., 2003).

Additionally, the fact that engagement of CD27 not only induces loss of its membrane expression but also the induction of effector functions corroborates the idea that CD27/CD70 interactions may be instrumental in generating effector T cells.

1.4. Strength of TCR signaling encompasses multiple components and can contribute for clonal selection

The strength of antigenic stimulation is a complex parameter that encompasses several factors: first, the number of APCs, the density of peptide-MHC complexes on their surface and the TCR avidity for the MHC-peptide complex, which together establish the antigen dose and determine the rate of TCR triggering; second, the density of co-stimulatory molecules, which determine the degree of signal amplification; and, finally, the duration of the APC-T cell interaction, which defines the extent of the signaling process (Lanzavecchia and

Sallusto, 2002). Collectively, these factors can make signal strength vary in several orders of magnitude, stimulating lymphocytes over a broad spectrum of conditions. Moreover, they can balance one another; for instance, a high level of co-stimulation might compensate a short-time APC-T cell contact, or a prolonged TCR stimulation might compensate for a low level of antigen.

The threshold of activation is not the same for all populations of T cells. This results from the different coupling status of the signaling machinery (Lanzavecchia and Sallusto, 2000). In naïve T cells, the components of the signaling apparatus are delocalized from the TCR and co-receptors. Upon antigen recognition, the kinases and adaptor proteins that mediate the signaling cascade initiated by TCR triggering are redistributed and associate preferentially with the proteins located on the membrane (Bachmann et al., 1999b). For this reason naïve T cells can take up to 20 hours of stimulation to be able to progress in the cell cycle. Co-stimulation can help to decrease the threshold of activation of naïve cells (Iezzi et al., 1998; Viola et al., 1999). Primed T lymphocytes, on the contrary, have the signaling machinery fully coupled and can be activated when stimulated for only 30 minutes or less, even in the absence of co-stimulation (Iezzi et al., 1998). In addition, memory cells are in a more advanced G1 phase of the cell cycle than naïve lymphocytes, expressing higher levels of active complexes of cyclin D and cyclin dependent kinase-6, a molecular complex crucial for progression through G1 and S phases of the cell cycle. The presence of high amounts of these molecular complexes in the cytoplasm is believed to confer to memory T cells a rapid cell cycle progression after re-stimulation and a lower threshold of activation than naïve cells (Veiga-Fernandes and Rocha, 2004).

The capacity of each of the numerous clones activated in a primary immune response to achieve effective stimulation is strictly dependent on TCR affinity for antigen and on the capacity to compete with other cells, of the same or of other clones, for limiting resources, such as APCs, antigen and also cytokines. As these variables are important components determining the overall signal strength, cells expressing TCRs with different affinities receive dissimilar levels of stimulation and will likely have different activation thresholds. Interestingly, there is evidence that competition for antigen between T-cell clones occurs in human populations of T cells. A recent study reports that intrinsic properties of TCR avidity for antigen co-segregate with distinct $CD8^+$ T-cell clonotypes in the context of EBV infection (Price et al., 2005). Indeed, while dominant clonotypes exhibit high levels of TCR

avidity directly *ex vivo*, as well as more sensitive functional response profiles, subdominant clonotypes show the inverse behavior. This suggests that TCR-dependent mechanisms could determine the optimization of the response during antigen-driven $CD8^+$ T-cell expansion in chronic infection. Although the relationship between antigen avidity and functional sensitivity is nonlinear, such progressive evolution toward high avidity $CD8^+$ T-cell usage amongst peripheral T-cell repertoire is potentially advantageous in terms of improved effector function and control of viral replication (Alexander-Miller et al., 1996; Derby et al., 2001; Sedlik et al., 2000). In addition, another study concluded that an affinity threshold mechanism should operate during the peripheral selection and expansion of antigen-specific $CD4^+$ T-cell populations to limit the competitive advantage of clonotypes with the highest avidity and prevent monopolization of the response (Malherbe et al., 2004). Such mechanisms would be of fundamental value to restrain proliferation of self-reactive T-cell clones with excessively high avidity that had escaped thymic selection.

1.5. Signal strength determines T-cell fate

The duration and strength of TCR stimulation are two important parameters that determine the cell fate. Interaction between one particular TCR and a MHC-complex lasts only for few seconds and can trigger some effector functions, such as killer T-cell execution of target cells. However, the stabilizing effect of the immunological synapse allows T lymphocytes to stay in contact with the APC for several hours. This favors the interaction of multiple TCRs on the same lymphocyte with one single MHC-complex, sustaining the signaling necessary to induce and maintain some more complex functions, like proliferation (Timmerman et al., 1996). High levels of activation, however, can be deleterious in the way that T cells can be driven to undergo a process called activation-induced cell death (AICD) (Alexander-Miller et al., 1996; Lenardo et al., 1999). This process can be circumvented to some extent by up-regulation of the anti-apoptotic factor Bcl-X_L, achieved by co-stimulation (Boise et al., 1995).

After priming, T lymphocytes are committed to proliferate, generating large numbers of antigen-specific cells. Following the clonal-expansion phase, a large-scale, programmed apoptotic episode occurs, resulting in a substantial reduction

in the number of antigen-specific T cells (Badovinac et al., 2002). The survival capacity – or "fitness" – of the lymphocyte sub-population that have escaped death results from the balance between resistance to cell death and responsiveness to homeostatic cytokines, like interleukin-7 and interleukin-15 (IL-7 and IL-15, respectively). T-cell survival is an important aspect that determines the extent of cell accumulation and, thus, the effectiveness of the immune response. Activation of T lymphocytes induces a marked raise in metabolic activity and mitochondrial volume, which are sustained by TCR and cytokine signaling that promote nutrient uptake and up-regulation of anti-apoptotic factors, such as Bcl-X_L. On antigen withdrawal, loss of signaling through TCR and cytokine receptors reduces nutrient uptake and elicits expression of pro-apoptotic molecules. Therefore, in the course of the immune response antigen withdraw renders lymphocytes more susceptible to cell death and, in order to maintain their high metabolic activity in the absence of antigen, survival signals become strongly required (Plas et al., 2002). IL-7 and IL-15, two constitutively expressed cytokines which sustain T-cell homeostasis, can be the source of such signals (Ma et al., 2006). Lymphocyte survival will, thus, depend on the expression of the corresponding receptors, together with the expression of anti-apoptotic factors. Interleukin 2 (IL-2), interestingly, is a cytokine expressed mainly on the antigen-driven phase and might play an opposite role to IL-15 in the control of memory T lymphocyte homeostasis (Ku et al., 2000).

Lymphocyte fitness was reported to be strongly dependent on signal strength. Experiments *in vitro* and *in vivo* have demonstrated that a weak TCR stimulation leads to defective proliferation in response to homeostatic cytokines and cell death by neglect, whereas a stronger stimulation enhances T-cell survival and cytokine responsiveness. Two mechanisms implicated in lymphocyte survival were identified: (1) up-regulation of Bcl-X_L and (2) expression of the IL-15Rα and γ_c chains, both improved in strongly stimulated T cells (Gett et al., 2003).

Another fundamental consequence of lymphocyte activation is acquisition of effector functions, a phenomenon also called differentiation. Interestingly, differentiation of lymphocytes might also be strongly dependent on signal strength. *In vitro* stimulation of naïve $CD4^+$ T cells with a weak stimulus can induce proliferation, but not further differentiation. Upon *in vivo* transfer these cells migrate to lymph nodes, proliferate and acquire effector functions. In contrast, naïve $CD4^+$ T cells that have been stimulated with a stronger stimulus in the presence of IL-12 or IL-4, which drive T cell polarization towards a T_H1 or T_H2

phenotype, respectively, upon *in vivo* transfer are excluded from lymph nodes and migrate directly to peripheral tissues to execute effector functions. *In vitro* experiments with naïve $CD8^+$ T cells have put in evidence that differentiation of these cells, similarly to $CD4^+$, is also dependent on signal strength, as $CD8^+$ T cells primed by a weak stimulus are committed to proliferation, but cells fail to up-regulate anti-apoptotic molecules and the receptors for homeostatic cytokines. Accordingly, when transferred to a syngeneic host these cells do not survive (Gett et al., 2003). Moreover, $CD8^+$ T cells given a sub-optimal stimulation fail to mediate cytotoxicity *in vivo*, a phenomenon that can not be overcome by transgenic expression of anti-apoptotic molecules (van Stipdonk et al., 2003).

Collectively, these evidences point to the strength of the signals delivered by TCR and cytokine receptors as being fundamental factors that define T-cell fate. This led to the formulation of a theory for lymphocyte differentiation generally known as «progressive differentiation model», which will be discussed in further detail later in this manuscript.

1.6. Lymphocyte differentiation and the pivotal role of cytokines

Differentiation of T lymphocytes following antigen stimulation is a process that relies on transcriptional programs controlling the cell cycle, response to cytokines, migratory properties, effector function and survival capacity. Signals provided by the TCR and cytokine receptors synergize to induce specific transcription factors that mediate chromatin remodeling events, leading to the expression of specific target genes. According to the progressive differentiation model (described in detail in section 3.2), while some transcriptional programs would be activated at low levels of stimulation, others would require a higher strength of stimulation and further signaling through cytokine receptors.

Cytokines play a crucial role in lymphocyte differentiation. These molecules are generally cell-secreted proteins that affect the properties or behavior of other cells upon recognition by a specific receptor. Some cytokines produced by T cells are also called interleukins (IL), some of which were already mentioned. The recognition of pathogens can induce DCs, macrophages, T cells and other players in the immune response to produce cytokines. The overall balance of the cytokines produced is, thus, determined by the differential interaction of pathogens

with the cells of the immune system and has a strong impact on the fate of such cells.

1.6.1. $CD4^+$ T lymphocyte commitment into T_H1 or T_H2 cell types

The polarization of $CD4^+$ T lymphocytes towards a T_H1 or a T_H2 phenotype, for instance, is a phenomenon profoundly influenced by cytokines present throughout the initial proliferative phase of T-cell activation. T_H1 and T_H2 subsets develop from common T-cell precursors, rather than from distinct lineages (Abbas et al., 1996). These precursors are mature, naive $CD4^+$ T lymphocytes that generally secrete IL-2 upon initial encounter with antigen. IL-2 is, thus, the earliest cytokine to be secreted during CD4 T helper polarization. IL-12 and IFN-γ, mainly produced by APCs in response to virus and some intracellular bacteria, as well as by activated T cells, drive $CD4^+$ T lymphocytes to develop into a T_H1 phenotype. IL-18 can also influence T_H1 development by increasing IFN-γ production and proliferation of T_H1 clones. An important impact on inducing $CD4^+$ T-cell proliferation was recently reported for IFN-γ (Whitmire et al., 2005a), which contrasts with other published data indicating a suppressive role for this cytokine in T-cell responses (Badovinac et al., 2000; Dalton et al., 2000; Refaeli et al., 2002). The signature cytokine of T_H1 subset is IFN-γ, but these cells secrete also large amounts of IL-2 and TNF-α and TNF-β. As a consequence of the cytokines they produce, T_H1 lymphocytes are extremely efficient in eliminating intracellular pathogens, like *Leishmania*, via macrophage activation. In result, T_H1-dominant immune responses are often associated with inflammation and tissue injury, the typical inflammatory reaction being delayed-type hypersensitivity (DTH) responses. Conversely, development of T_H2 $CD4^+$ T cells is promoted by IL-4 and IL-6. The T_H2 $CD4^+$ T subset secretes the cytokines IL-4, IL-5, IL-10 and IL-13, which accomplish for humoral immunity to helminthic parasites and are responsible for immune responses to persistent antigens, like allergens. In particular, IL-4 is the main inducer of B-cell switching to IgE production and is therefore a key initiator of IgE-dependent, mast-cell-mediated reactions; IL-5 is the principal eosinophil-activating cytokine, and mice deficient for IL-5 or its receptor show marked defects in eosinophil responses to helminthes; IL-4 and IL-13 antagonize the macrophage-activating action of IFN-γ; IL-10 suppresses numerous

macrophage responses; and transforming growth factor β (TGF-β), a cytokine produced by some T_H2 cells and many other cell types, is anti-proliferative and inhibits leukocyte activation; finally, T_H2 cells are excellent helpers for B lymphocytes, as they stimulate the production of high levels of IgM and non-complement-fixing IgG isotypes, such as IgG_1 in mice, or its homologue, IgG_4 in humans (Abbas et al., 1996; Glimcher and Murphy, 2000; Mosmann and Sad, 1996; O'Garra, 1998).

In addition to the above described subsets of $CD4^+$ T lymphocytes, individual cells that do not exhibit a cytokine-secreted pattern that fits into the T_H1 or T_H2 classification can often be found, especially in humans. These cells secrete a complex and heterogeneous set of cytokines with various combinations of IL-2, IL-4, IL-5 and IFN-γ, and sometimes IL-10 and TGF-β, which can accomplish for important functions but are not characteristic of either subset. Such mixed cytokine-producing cells have been classified as T_H0 (Abbas et al., 1996).

The T_H1 and T_H2 secreted cytokines endow the corresponding $CD4^+$ T subsets with the capacity of cross-regulate each other's activity and development. For instance, IFN-γ produced by T_H1 cells amplifies T_H1 development and prevents proliferation of T_H2 cells, whereas IL-4 and IL-10 inhibit the generation of T_H1 cells. Therefore, the balance between T_H1 and T_H2 secreted cytokines determines the extent of polarization and the type of the prevalent immune response. Accordingly, when a T-cell immune response begins to develop along one pathway, namely T_H1 or T_H2, it tends to become progressively polarized in that direction. Furthermore, a regulatory function for T_H2 subset has been proposed, in which these cells, owing to the anti-inflammatory cytokines they secrete, would help to limit the injurious consequences of T_H1-mediated protective immunity. This hypothesis is based on the fact that, during *in vitro* T-cell activation, T_H1 precede the development of T_H2 responses (Croft and Swain, 1995) and predicts that T_H1 responses would develop predominantly in early time points, when antigen concentration is not too high and the major APCs are DCs and macrophages, which secrete large amounts of IL-12, while T_H2 committed lymphocytes would appear later in the response, when antigen doses become higher and antigen can be presented by APCs that do not produce IL-12, thus favoring T_H2 polarization (Abbas et al., 1996).

Expression of cytokines in $CD4^+$ T helper cells is transient; after primary activation cytokines are expressed for several days (Assenmacher et al., 1998)

and, in later stages of the response, expression of cytokines is memorized. In result, upon re-stimulation, polarized CD4 T cells recall the expression of cytokines they had been instructed to express earlier (Murphy et al., 1996). One of the reasons that explains cytokine memory is the somatic epigenetic imprinting of the corresponding genes (Agarwal and Rao, 1998; Bird et al., 1998; Bix and Locksley, 1998). Indeed, the cytokine genes that have been previously expressed in differentiated $CD4^+$ T cells remain accessible, rendering possible their later expression after recall stimulation, whereas others remain silenced (Rengarajan et al., 2000).

At the molecular level, differentiation of $CD4^+$ T cells is orchestrated by up-regulation of specific transcription factors. Two key transcription factors are involved in helper T-cell polarization: T-bet, which induces T_H1 lineage development, and GATA3, which drives polarization towards a T_H2 phenotype (Szabo et al., 2000; Zheng and Flavell, 1997). T-bet initiates T_H1 lineage development from naive unpolarized cells both by activating T_H1 genetic programs and by repressing the opposing T_H2 programs. GATA3, in turn, activates the expression of T_H2 prototypic genes, such as IL-4, IL-5, IL-6, IL-10 and IL-13, but does not abort the program for T_H1 cytokine gene expression, as IFN-γ gene expression remains unaltered. Therefore, it is likely that other factors implicated in T_H1 cytokine expression, like Stat-4, ERM, class II MHC transactivator (CIITA) or Hlx, are lost during T_H2 polarization, just as *gata-3* expression is lost in T_H1 cells. In addition to GATA3, other transcription factors involved in T_H2 differentiation were identified, including Stat-6, c-Maf, NF-IL6, NF-AT and AP-1. The precise contribution of each of these molecules, however, remains to be elucidated (Murphy et al., 2000; Szabo et al., 2000; Zheng and Flavell, 1997).

Expression of T-bet and GATA3 is up-regulated not only by the action of polarizing cytokines, but also by signaling through the TCR (Iezzi et al., 1999; Richter et al., 1999). Indeed, it had already been reported that the extent of T helper polarization can be influenced by the strength of TCR stimulation, as well as by the level of co-stimulation. In general, low antigen concentrations and conditions of low co-stimulation tend preferentially to induce T_H1 responses, whereas high doses of antigen and co-stimulation potentiate T_H2 development (for a review, see Abbas et al., 1996; Glimcher and Murphy, 2000). One possible explanation is that high levels of antigen lead to repeated TCR stimulation, thus increasing IL-4 production and T_H2 development, or induce a state of

immunological unresponsiveness, which in general shuts off T_H1 responses (De Wit et al., 1992). In the same line of reasoning, another possibility is that high co-stimulation would promote T_H2 responses by increasing the magnitude of the initial T-cell activation which, again, boosts IL-4 production and favors the IL-4-dependent autocrine pathway of T_H2 differentiation. T_H1 polarization, in the other hand, relies on IL-12 secreted by APCs in parallel with some level of co-stimulation (Murphy et al., 1994), but is less influenced by the magnitude of the TCR signaling. Interestingly, despite high levels of co-stimulation by APCs enhance T_H2 development, fully differentiated T_H2 cells respond to antigen without co-stimulation, whereas T_H1 cells still require co-stimulation in order to become activated (McKnight et al., 1994; Seder et al., 1994). Finally, it has also been proposed that at high signal strength conditions T-cell polarization may possibly be accomplished even in the absence of cytokines (Lanzavecchia and Sallusto, 2002).

Lastly, it should be stressed out that the complete activation of naïve T cells is accompanied by profound changes in the homing behavior of these cells. Lymphocytes can modify their migration properties due to changes in the cell-surface molecules, such as chemokine receptors and adhesion molecules (Bonecchi et al., 1998; Sallusto et al., 1998; Siveke and Hamann, 1998). Hence, while some lymphocytes keep the potential to home to the lymph nodes (by maintaining the expression of CCR7 and L-selectin), where they might be re-stimulated by antigen and/or by survival-promoting cytokines, others acquire the capacity to migrate to inflamed peripheral tissues where they can finish their differentiation process or exert immediate effector functions. Interestingly, effector cells can be found in non-lymphoid tissues for prolonged period of time in a non-dividing state (Reinhardt et al., 2001). However, whether these cells are long-lived or replaced over time remains to be clarified.

1.6.2. $CD8^+$ T lymphocytes differentiate into cytotoxic T cells

TCR recognition of peptide–MHC class I complex on naïve $CD8^+$ T cells, in conjunction with co-stimulatory and helper signals, invoke the typical events of an immune response that eventually culminate in the generation of memory: cellular activation, clonal expansion, differentiation into effector cytotoxic T lymphocytes

(CTLs) and contraction. $CD4^+$ T-cell help is not always essential for CTL differentiation in primary immune responses, since activation of naïve $CD8^+$ T cells can be often achieved when lymphocytes are activated *in vivo* by viruses, peptide-pulsed DCs or tumor cells in the absence of $CD4^+$ T cells (Dalyot-Herman et al., 2000; Marzo et al., 2000; Marzo et al., 2004). These $CD4^+$ T cell-independent CTL responses concern only the primary immune responses and occur especially in non-limiting conditions, such as high antigen-specific precursor frequency, high TCR affinity for antigen or a strong innate immune response. Conversely, in limiting conditions CTL responses are strongly dependent on $CD4^+$ T-cell help and also on co-stimulation via CD28 (Zhan et al., 2000).

Originally, help for the primary $CD8^+$ T-cell response was thought to be mediated by cytokines secreted by $CD4^+$ T helper cells, such as IL-2, which promotes $CD8^+$ T cell proliferation *in vivo* (Keene and Forman, 1982). Later, $CD4^+$ T-cell help has been shown to involve CD40–CD40L interactions between APCs and $CD4^+$ T cells, leading to the APC activation required for the priming of naïve $CD8^+$ T cells (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). CD40L is a cell-surface molecule transiently expressed by recently activated $CD4^+$ T cells which is involved in their activation and development of effector functions (Banchereau et al., 1994). More recently, a further interaction has been demonstrated between $CD8^+$ and $CD4^+$ T cells via CD40-CD40L (Bourgeois et al., 2002a). In particular, it has been shown that mRNA coding for CD40 increases in T cells shortly after activation and CD40 protein expression is transiently induced at the surface of $CD8^+$ specific T cells during the first days of the response. Moreover, when antigen specific $CD8^+$ T lymphocytes, but not DCs, lack CD40 defects in proliferation and cytokine production capacity were also observed, indicating $CD4^+$ T cells can possibly deliver the helper signal via CD40L directly to $CD8^+$ T cells expressing CD40 (Bourgeois et al., 2002a).

Notably, $CD4^+$ T-cell help was reported to be not essential for the initial activation, expansion and development of effector functions of naïve $CD8^+$ T lymphocytes. However, memory $CD8^+$ T cells generated in the absence of $CD4^+$ T-cell help exhibit poor proliferative capacity and cytokine production upon *in vitro* re-stimulation (Bourgeois et al., 2002a; Janssen et al., 2003). The requirement for $CD4^+$ T cells is apparently transitory, as *in vivo* depletion three days following priming have no deleterious effect on the generation of functionally competent memory $CD8^+$ T cells (Janssen et al., 2003). Moreover, unaffected memory $CD8^+$

T cell responses can be generated in the absence of $CD4^+$ T cells if help was provided during the primary response (Bourgeois et al., 2002b; Shedlock and Shen, 2003; Sun and Bevan, 2003). Together, these evidences indicate that $CD4^+$ T cell help is fundamental for effective secondary responses, but it is only required during the initial stages of activation. Thus, imprinting for memory potentiality would be attributed during priming and would have a distinct nature from the signals that dictate the genesis of competent cytotoxic effector cells.

The transition from naïve to effector and memory $CD8^+$ T-cell populations is accompanied by marked and heritable changes in gene expression (Kaech et al., 2002). Likewise $CD4^+$ T cells, after priming the naive $CD8^+$ T-cell precursors undergo genetic remodeling events that result in the expression of genes fundamental to $CD8^+$ effector T-cell function, including genes that encode cytokines and chemokines, as well as genes associated with cytolysis. IFN-γ and TNF-α are the main cytokines induced following $CD8^+$ T-cell activation but the hallmark of activated $CD8^+$ T lymphocytes is the expression of cytolytic molecules, such as perforin, granzymes or Fas ligand (FasL).

IFN-γ production by cytotoxic $CD8^+$ T lymphocytes is a pivotal mechanism in the combat to viral infections. IFN-γ is a direct inhibitor of viral replication and facilitates antigen processing of viral peptides by inducing expression of MHC class I molecules, TAP (standing for transporter associated with antigen processing) transporter proteins and components of the proteasome, a protease complex responsible for degradation of cytosolic proteins, such as viral proteins, and the generation of peptides that can be presented at the cell surface by MHC class I molecules.

Unlike naïve cells, which produce little IFN-γ, effector $CD8^+$ T cells secrete large amounts of IFN-γ immediately after activation. The transition from naïve to effector $CD8^+$ T-cell stage is controlled, at least in part, by the transcription factor T-bet. Expression of T-bet is induced early after T-cell priming by signaling through the TCR and also the IFN-γ receptor (IFN-γR). Similarly to $CD4^+$ T cells and NK cells, which both require T-bet for control of IFN-γ production, expression of IFN-γ by antigen-specific $CD8^+$ T lymphocytes is also dependent on expression of T-bet (Sullivan et al., 2003; Szabo et al., 2002). Moreover, in the absence of T-bet, $CD8^+$ T cells fail to acquire a normal profile of effector cell-surface markers, such as downregulation of CD62L, an event associated with differentiation and activation. Curiously, these changes are masked if the T cells are activated in an antigen

non-specific manner (Sullivan et al., 2003), which suggests that the strength of the signal transduced by the TCR might play an important role. Finally, T-bet is also implicated in the development of cytotoxic effector functions, as T-bet deficient mice exhibit defective *in vivo* killing when compared to wild-type mice and fail to mount a protective response against LCMV infection (Sullivan et al., 2003). Nevertheless, the cell killing capacity was not completely abrogated in the absence of T-bet, demonstrating that other factors might have a role in this setting. There are also evidences indicating that, in the absence of T-bet, an intrinsic defective generation of viral-specific effector $CD8^+$ T cells might occur (Glimcher et al., 2004; Juedes et al., 2004).

The T-box transcription factor eomesodermin (Eomes), a paralogous of T-bet, was recently identified as having a complementary action to T-bet in the development of cytolytic effector function and in the long term renewal of memory $CD8^+$ T cells (Intlekofer et al., 2005; Pearce et al., 2003). Eomes was originally known by its important role in development, for the determination of mesodermal-cell fate in vertebrates. More recently, it was demonstrated that Eomes is expressed at low levels in naïve $CD8^+$ T cells and that mRNA levels are markedly increased upon activation. As homozygous Eomes deficient mice die early in embryogenesis, the relative contributions of Eomes and T-bet in $CD8^+$ T lymphocyte effector differentiation and function was evaluated using transfectant retroviruses that encode a dominant negative form of T-bet (DN T-bet) or Eomes (DN Eomes) and mice with compound mutations of T-bet and Eomes genes. In the absence of both T-bet and Eomes, IFN-γ production was shown to be severely compromised. Moreover, introduction of DN Eomes or DN T-bet (which targets both T-bet and Eomes) into $CD8^+$ T cells from wild-type mice led to a greater defect in granzyme B induction than did gene deletion of T-bet alone, suggesting that Eomes might mediate an alternative, complementary pathway to T-bet for the induction of cytotoxic genes (Pearce et al., 2003). Indeed, inhibition of Eomes in activated lymphocytes lead to a substantial defect in killing capacity *in vitro*. In addition, haplo-insufficiency of Eomes ($Eomes^{+/-}$) results in reduced levels of perforin mRNA, but IFN-γ transcripts are only minimally affected, which might be due to the partial knock-down and/or to the presence of normal levels of T-bet in these cells. Ectopic expression of Eomes by T-bet deficient $CD8^+$ T cells can restore IFN-γ production, indicating that the regulation of IFN-γ transcription might be under the control of both Eomes and T-bet. Additionally, Eomes over-

expression in $CD4^+$ T_H2 polarized cells, a cell lineage normally devoided of cytolytic function, was shown to be sufficient to induce IFN-γ and cytotoxic gene expression (perforin and granzyme B). Furthermore, expression of Eomes correlates with the expression of the mRNA for the lytic molecules perforin and granzyme B in activated NK cells and $CD8^+$ T cells. Together, these evidences indicate that Eomes not only controls IFN-γ production, but also appears to be fundamental for the expression of perforin and granzyme B by effector cells (Glimcher et al., 2004; Pearce et al., 2003).

In addition to regulate cytotoxic programming, Eomes and T-bet seem to be cooperatively responsible for the responsiveness of primed cells to IL-15 by inducing expression of CD122, the receptor that confers the ability to respond to IL-15. Both T-box factors are necessary and sufficient to induce expression of CD122 in naïve cells that are differentiating into effector cells and also for maintaining high levels of CD122 expression in mature memory cells. Furthermore, loss of T-bet in Eomes haplo-insufficient mice renders the peripheral lymphoid organs devoided of IL-15-dependent lineages, a phenotype alike to IL-15 deficiency (Intlekofer et al., 2005). However, the defect in cytotoxic function in compound T-box factor mutants is not shared by IL-15 pathway mutants, indicating that IL-15 responsiveness and effector function may be programmed in parallel by Eomes and T-bet.

In conclusion, Eomes seems to complement the role of T-bet in governing cellular immunity by providing redundancy and, possibly, cooperativity in the induction of effector genes of T cells, as well as in the ability of memory cells to undergo IL-15-driven proliferative renewal. It would be interesting to investigate whether other memory related cellular events, such as signal transduction, survival and apoptosis, cell cycle regulation, etc, are also under the control of Eomes and T-bet. Moreover, the precise mechanisms by which these T-box factors control $CD8^+$ effector T-cell development and IL-15 responsiveness remain to be elucidated.

2. CD8$^+$ T-cell mediated cytotoxicity

The main function of CD8$^+$ T lymphocytes is the killing of infected cells. Lymphocyte-mediated cytotoxicity uses two alternative mechanisms to induce target cell death (Russell and Ley, 2002). The first one is a pathway initiated by FADD (Fas-associated via death domain) upon engagement of a target cell receptor by CTLs. Evidences from gene knock-out studies indicate Fas as the most physiologically important initiator of FADD pathway, although other receptors of the TNF receptor family, such as TNFR1 and TRAILR, can also mediate cell death by activation of this pathway (Kischkel et al., 2000; Sprick et al., 2000). The second killer mechanism is the localized exocytosis of cytolytic granules containing perforin and granzymes. Perforin is a protein that can polymerize to form membrane pores, which potentiates the traffic of granzyme proteins into the target cells where they subsequently initiate cell death through various mechanisms, including activation of caspase-independent mitochondrial and nuclear cell-death pathways, disruption of the plasma membrane and damaging of the DNA. The granule-exocytosis pathway encompasses several alternative mechanisms that depend on the spectrum of granzymes induced upon activation. This is, indeed, the crucial pathway for mediating cellular cytotoxicity against intracellular pathogens and tumors, although undoubtedly FADD-activating receptors and the secretion of cytokines are also important (Balkow et al., 2001; Muller et al., 2003b).

Both FADD and perforin/granzymes pathways require previous activation of the naïve CTL precursors in order to be induced. The same signals that instruct the lymphocyte to proliferate and become activated also induce de novo synthesis of Fas ligand (FasL) and cytotoxic granules. These armed CTLs can act very rapidly upon encountering specific antigen on other cells. Once formed, the granules can be reoriented and released within minutes of TCR stimulation into the region of contact between the target cell and the CTL, in the vicinity of the TCR activation cluster. Whether the architecture of the immune synapse has a physiological function in the reorientation and exocytosis of the cytotoxic granules remains poorly defined (O'Keefe and Gajewski, 2005). Activity of FasL has a much slower kinetics, since very little amounts of the molecule can be stored in the cytoplasm, even in activated cells, and induction of new ligand demands 1-2 hours of TCR stimulation. On the other hand, FasL has a long half-life (2-3 hours) on the

cell surface, which enables the effector cell to maintain cytotoxic activity even after TCR stimulation has ceased. In consequence, all the cells that express Fas in the surrounding area can be promiscuously killed, even those that do not express the antigen recognized by the TCR – a phenomenon known as bystander killing. Hence, the target killing via Fas can be much more promiscuous than cytotoxicity mediated by perforin and granzymes, once the vectorial release of granules to the contact site between the killer and target cells ensures exquisite specificity to the perforin-initiated killer pathway (Russell and Ley, 2002).

2.1. FADD pathway

Mechanisms of $CD8^+$ T-cell mediated cytotoxicity induce cell death by activating apoptotic pathways on the target cells. These pathways basically consist of proteolytic cascades and commonly involve the activation of a particular type of protease cascade, the caspase cascade. These enzymes have a cysteine residue in the active site and mediate a specific cleavage at aspartic acid residues. The substrates of the various caspases are ultimately responsible for the morphological (cytoskeletal, nuclear membrane breakdown) and biochemical (DNA laddering) changes associated with apoptosis.

In the case of Fas, interaction with the ligand recruits FADD to the cytoplasmic domain of the receptor. FADD interacts with caspase-8 through the corresponding death effector domains, which somehow induces caspase-8 activation by cleavage of an inhibitory pro-domain. The active caspase is able to process downstream pro-caspases, as well as its own pro-caspase precursor. Caspase activation proceeds in a hierarchical manner throughout a series of proteolytic cleavage events of specific targets that culminates in activation of effector caspase-3, caspase-6 and caspase-7. It is finally the action of these molecules that leads to the proteolytic cleavage of the various cellular substrates responsible for the particular DNA fragmentation that characterizes apoptosis (Dempsey et al., 2003).

Activation of the caspase cascade is referred to as type 1 apoptosis. An alternative type of apoptosis, called type 2 apoptosis, involves loss of organelle function. Signaling initiated by FADD, together with activity of Bcl-2 family proteins, plays a major role in regulating mitochondrial dysfunction on this second type of apoptosis. Bcl-2 and Bcl-X_L are anti-apoptotic molecules that sequester pro-

apoptotic proteins containing the homology motif BH3 (from Bcl-2 homology domain), such as Bak. The oligomerization of Bak at the surface of the outer mitochondrial membrane is thought to form intramembranous pore complexes that allow the release of cytochrome *c* to the cytosol. This process is, in turn, dependent on the proteolytic processing of Bid by activated caspase-8. The cleavage of Bid releases the potent pro-apoptotic truncated protein tBid that localizes at the outer mitochondrial membrane and drives the assembly of Bak proteins. The release of cytochrome *c* induces the oligomerization of apoptotic protease activating factor-1 (Apaf-1), a protein that interacts with caspase-9 and promotes, in the presence of ATP, the generation of a functional apoptosome. The apoptosome complex can cleave and activate procaspase-3 and, from this point on, the subsequent apoptotic events follow the same pathway as the one initiated by caspase-8. Thus, regulation of cytochrome *c* release by the receptor Fas is a balance between the activation of Bid and the inhibition of Bcl-2 related molecules (Dempsey et al., 2003).

In contrast to Fas, TNFR1 (the main receptor for TNF-α) only signals for cell death in certain circumstances, like when protein synthesis is blocked. In this case, TNFR1 associates with TRADD which can interact with the death domain of FADD, thereby initiating the caspase-8 activation pathway. Nonetheless, TNFR1 engagement is more likely to induce the activation of inflammatory genes. In this situation TRADD recruits TRAF1, TRAF2 and RIP, leading to the activation of the NFκB and JNK signaling pathways which initiate inflammatory responses and protect the cell from apoptosis (Dempsey et al., 2003).

The relative contribution of FADD and granule exocytosis pathways for the control of intracellular infections, the main function of immune responses mediated by $CD8^+$ T cells, has been analyzed using genetically deficient animals (reviewed by Russell and Ley, 2002). Indeed, each pathway assumes a different importance depending on the type of infection. Perforin is, in general, more important than the Fas pathway for clearance of pathogens, and abrogation of both mechanisms usually results in uncontrolled infection as, for instance, with neurotropic murine hepatitis virus. In this infection, perforin deficiency significantly delays viral clearance from the central nervous system, but absence of Fas has no effect, unless perforin is also absent. Interestingly, complete clearance of this infection requires IFN-γ. One possible explanation is that IFN-γ might up-regulate the expression of MHC class I or Fas on oligodendrites, thus increasing their

sensitivity to CTLs. Another example is infection with murine cytomegalovirus, which is absolutely dependent on perforin, but not Fas, for complete virus clearance during acute infection. However, apoptosis mediated by Fas is important in limiting chronic inflammatory disease.

2.2. Perforin/granzymes pathway

Antigen recognition by the TCR on $CD8^+$ T lymphocytes activates transcriptional mechanisms that eventually lead to the production of cytotoxic granules and their constituent proteins. Concretely, cytotoxic granules are specialized lysosomes composed of perforin, granzymes and other lysosomal proteins, such as cathepsins, granulysin and calreticulin, which begin to be synthesized within one day after T-cell activation then become resident in the cytoplasm. Specific signals generated upon activation endow the secretory machinery of effector lymphocytes to direct the lytic granules to the site of contact with the target cell. Here, the granule fuses with the plasma cell membrane, and its contents are secreted into the tight intracellular junction formed between the two cells (Trambas and Griffiths, 2003).

2.2.1. Components of the granules

Perforin

Perforin is a pore-forming protein that polymerizes on the plasma membrane in a Ca^{2+}-dependent manner. Originally, this phenomenon was considered to induce direct damage to the plasma membrane, causing cell lysis identically to the complement. Nevertheless, it has been shown that perforin-induced membrane damage is not sufficient to cause apoptosis (Duke et al., 1989). Granzymes or other granule components are required to act in cooperation with perforin to transduce the apoptotic signal. Homo-polymerization of perforin on the surface of the target cell forms transmembrane pore structures that can act as a channel. These perforin channels were thought to act as a gateway for delivery of granzymes into the cytosol of the target cell, thus implying that productive cytotoxicity depends on the simultaneous expression and exocytosis of perforin

and granzymes. However, this idea has been questioned since the size of this pore is probably too small to permit large molecules like granzymes to enter target cells (Lieberman, 2003). Apparently, the size of the pore formed in the plasma membrane largely depends on the amount of perforin molecules available. The effective concentration of perforin present in the contact region between the CTL and their targets is, however, not known. Nevertheless, despite the precise mechanism of how perforin delivers granzymes is still undisclosed, perforin is unquestionably required for CTL-mediated cytolysis via the granule exocytosis pathway since the gene knock-out results in severe immunodeficiency against virus and tumors (Kagi et al., 1994). An alternative hypothesis to explain granzyme entry suggests that perforin-induced pore formation generates a signal for the target cell to repair the damage by endocytosing the perforin and surrounding plasma cell membrane; granzymes in the vicinity of the lesion would be also endocytozed and ultimately delivered to the target cell cytoplasm and nucleus, where they initiate the apoptotic events (Podack, 1999).

Granzymes, cathepsins and calreticulin

Granzymes are serine proteases with high specificity for substrate. One can find two clusters of granzyme genes in the genome of mice and men: the tryptase granzymes A and K gene cluster and the granzymes B, C, D, E, F, G and H gene cluster. Additionally, a third gene cluster was identified in humans, encoding granzyme M along with neutrophil serine proteases (Lieberman, 2003). As granzymes are positively charged proteins, they are non-covalently bound by charge, together with perforin, to the acidic granule proteoglycan serglycin. Inside of the granule, the acidic pH maintains granzymes in an inactive state, preventing damage of the lymphocyte itself by its own granule enzymes. Moreover, at least some granzymes (e.g. granzyme B) are synthesized in an inactive pro-enzyme form. Only after proteolytic cleavage of the inhibitory domain by cathepsin C (also known as dipeptidyl peptidase I), another component of the cytotoxic granules, these granzymes become active. In addition, calreticulin, a Ca^{2+}-dependent chaperone protein that binds to perforin, is also present in the granules. This chaperone is thought to prevent perforin assembly in the granules, thus avoiding the exit of granzymes to the cytosol, which would led to autolysis of the CTL.

Another lysosomal protein present inside the cytotoxic granules is cathepsin B. This protein is thought to protect the CTL after exocytosis from its own cytotoxic molecules secreted into the synapse, by cleavage and inactivation of perforin (Balaji et al., 2002).

Granulysin

A further granule component protein called granulysin has been identified in human, but not rodent, CTLs and NK cells. Similarly to perforin and the granzymes, granulysin is synthesized upon T-cell activation and localizes in the same cytotoxic granule compartment (Krensky and Clayberger, 2005). Granulysin is an effective antimicrobial agent related with virulence factors of amoebae, which has the ability to kill both intracellular and extracellular bacteria, as well as mycobacteria and fungi, by increasing their membrane permeability. Granulysin was also shown to display cytotoxic activity against mammalian tumors *in vitro* (Gamen et al., 1998). The homology with pore forming proteins suggests that granulysin might use this strategy to induce damage in the target cell membrane. In this way, granulysin could provide a backup strategy for perforin-membrane disruption in humans. However, there is no experimental evidence supporting this idea. Granulysin can induce mitochondrial depolarization and the release of cytochrome *c* as well. Nevertheless, granulysin-induced mitochondrial damage does not cause procaspase 9 activation via the classical apoptosome, but it still manages to activate caspase 3. Evidences suggest that granulysin-mediated cell death might require the presence of mitochondria in the target cell, as red blood cells are not lysed by granulysin, but reticulocytes are sensitive (Li et al., 2005). Moreover, it has recently been shown that granulysin also acts as a chemoattractant for monocytes, $CD4^+$ and $CD8^+$ memory ($CD45RO^+$), but not naïve ($CD45RA^+$) T cells, NK cells, and mature, but not immature, monocyte-derived DCs. Furthermore, granulysin also induces the production of chemokines, including MCP-1 and RANTES, and cytokines, such as IFN-γ by monocytes. Thus, in addition to eliminating pathogens and tumor cells, granulysin helps to recruit immune cells to the sites of inflammation (Deng et al., 2005). Since a murine orthologue of this molecule has not yet been identified, the importance of granulysin for CTL function *in vivo* is still not completely understood. However, since most of the perforin-independent cytotoxic activity of CTLs in perforin

deficient mice is accounted by Fas, granulysin is expected to play a minor role in this setting.

2.2.2. The universe of granzymes

Target cell apoptosis induced by CTLs can be accomplished by both caspase dependent and independent pathways, depending on the acting granzyme set. For instance, granzyme A, C and granulysin trigger the caspase-independent pathway of cell death, but granzyme B can induce both the caspase-dependent and independent pathways of apoptosis (Lieberman, 2003).

Granzyme B

Granzyme B cleaves proteins preferentially after an aspartic acid residue, a common feature to caspases. Indeed, a vast number of procaspases can be cleaved and activated by granzyme B, such as procaspase-3, for instance. Cleavage of procaspases generally activates the downstream proteolytic cascade that culminates in the death of the target cell. Granzyme B was reported to activate other non-caspases substrates as well. The most important are Bid, which induces mitochondrial dysfunction (see above) and ICAD, the inhibitor of a nuclease known as caspase-activated DNAse (CAD) (Thomas et al., 2000). CAD is a nuclear protein that is retained in its inactive form by association with ICAD (inhibitor of CAD). Cleavage of ICAD in result of caspase-3 or granzyme B activity facilitates the assembly of CAD into its active form, which mediates oligonucleosomal fragmentation of DNA and, ultimately, leads to cell death (Sharif-Askari et al., 2001).

Granzyme B induces rapid DNA fragmentation of target cells, as demonstrated in granzyme B deficient mice whose CTLs are unable to induce rapid target cell DNA fragmentation *in vitro* (Heusel et al., 1994). Using mice deficient for granzyme B, granzyme A or both granzymes A and B, it has also been demonstrated that granzyme B plays an important role *in vivo*, in providing protection against the natural mouse poxvirus pathogen, ectromelia (Mullbacher et al., 1999).

Granzyme A

Granzyme A is the most abundant protease in the cytotoxic granules. Unlike granzyme B, it is also a component of the lytic granules of NK cells. Expression of granzyme A is activated approximately three to five days after naïve T cell priming and, in contrast to granzyme B and perforin, granzyme A continues to be expressed long after T-cell activation. By a poorly understood mechanism, upon delivery into the target cell along with perforin both granzymes A and B rapidly accumulate in the nucleus, where they can activate pathways of nuclear damage (Lieberman, 2003).

Cellular death mediated by granzyme A is completely independent of caspases. However, it elicits cellular events common to those induced by the caspase pathway, such as chromatin condensation, nuclear fragmentation, externalization of phosphatidyl serine in the cell membrane and loss of mitochondrial transmembrane potential. DNA damage in the granzyme A pathway is provoked by single-stranded nicks that originate large DNA fragments. The enzyme that mediates these events is called NM23-H1 and is inhibited by a protein, SET, that is a direct substrate of granzyme A and belongs to a recently described multiprotein complex. The SET complex is ubiquitously expressed and contains three granzyme A substrates: the nucleosome-assembly protein SET; the DNA bending high-motility group protein 2, HMG2; and the base excision repair (BER) enzyme apurinic/apirimidinic endonuclease 1, APE1 (Beresford et al., 2001). The cleavage of either of these proteins by granzyme A destroys all of their known functions. When granzyme A enters the target cells, the SET complex translocates to the nucleus in few minutes (Fan et al., 2003a). Cleavage of SET unleashes the inhibition of NM23-H1, which then begins DNA nicking. The SET complex is, therefore, fundamental for granzyme A-mediated DNA breakdown. The relevance of NM23-H1 and SET in this setting was demonstrated by overexpression of NM23-H1 and silence of SET, which both increase DNA damage and cell death, and by further experiments in which diminished cell death was observed in targets with silenced NM23-H1 or enhanced SET expression (Fan et al., 2003a). To date, the normal physiological function of the SET complex was not completely demonstrated, but the functions of its different components strongly suggest a role in the regulation of chromatin structure, integrity and gene expression. Oxidative stress and granzyme A loading into the target cell induces

rapid translocation of the SET complex from the endoplasmic reticulum, to which is generally associated, into the nucleus. This raised the hypothesis that SET complex proteins are involved in the repair response to oxidative damage (Fan et al., 2003b). Indeed, granzyme A activity was recently shown to induce loss of mitochondrial transmembrane potential in a Bid and caspase independent ways (Martinvalet et al., 2005). The mitochondrial attack is apparently direct, does not require the SET proteins and is insensitive to Bcl-2 over-expression and caspase inhibition.

In addition, granzyme A cleavage of APE1, a multifunctional repair protein, reinforces the apoptosis process initiated with activation of NM23-H1 by preventing cell recovery via repair (Fan et al., 2003b).

Granzyme A displays two further activities that contribute to target cell death. The first one is the disruption on the nuclear envelope and chromatin integrity by the cleavage of lamins. These filament proteins constitute the main structural components of the inner nuclear envelope and provide anchor sites for chromatin and the nuclear pore complexes. Lamin cleavage is also elicited by caspases, which suggests that disruption of the nuclear lamina might be a requisite for inducing apoptosis by any means (Zhang et al., 2001a). The second additional granzyme A activity is degradation of the linker histone H1, which anchors the DNA around the core histones (Zhang et al., 2001b). Removal of H1 histones opens up the chromatin and enhances DNA fragmentation by nucleases, which might account for the synergy action of granzymes A and B in the induction of target cell death.

Orphan granzymes

Granzymes of unknown function are referred as “orphans”. Apart from granzyme A and B, the granules also contain the granzymes H and K in humans and C, D, E, F, G, K and M in mice. Orphan granzymes are less expressed in CTLs than granzyme A and B. In mice, however, granzymes C, D, F and K have an important expression in NK cells, which might be related to specialized roles in the induction of target cell death in specific physiologic situations (Lieberman, 2003). Granzyme C has been reported to induce rapid cell death by a distinct pathway from that induced by either granzymes A or B (Johnson et al., 2003). Cell death associated events induced by granzyme C include cell-membrane damage,

single-strand DNA nicking, mitochondrial swelling, depolarization and release of cytochrome c, which occurs via an apparently caspase-independent mechanism. In addition, recent studies using mice with deficient expression of granzymes C and F reports that these enzymes might be implicated in the control of the gammaherpesviruses *in vivo* (Loh et al., 2004; Revell et al., 2005). For granzymes H and M, substrate specificity has been determined to be chymotrypsin-like, although specific protein substrates have not been identified, nor has a pro-apoptotic role yet been identified for granzyme H (Lieberman, 2003). Granzyme M is not expressed by conventional $CD4^+$ and $CD8^+$ T cells, but it is preferentially expressed by cells participating in innate immune responses, like NK cells and γδ T cells (Sayers et al., 2001). Recently granzyme M has been reported to induce perforin-mediated cell death by a unique mechanism not involving DNA fragmentation, caspase activation or mitochondrial involvement (Kelly et al., 2004) Additionally, a regulatory function was proposed to granzyme M, which was shown to cleave and inactivate the proteinase inhibitor 9, an endogenous inhibitor of granzyme B (Mahrus et al., 2004).

It is possible that multiple granzymes activating distinctly different pathways to cell death provide alternative ways to induce apoptosis of target cells. This could be of great value if pathogens develop strategies to elude immune surveillance that directly inhibit specific granzymes. In addition, the fact that mice have evolved a more extended set of granzymes possibly reflects an evolutionary backup for protection from a more diverse set of microbial pathogens than that encountered by humans (it seems unlikely that selection pressure was provided by tumors, given the short reproductive cycle of mice).

2.3. Other molecules expressed by $CD8^+$ T cells

2.3.1. TGF-β

The TGFB1 (herein simply called TGF-β) is a pleiotropic regulatory cytokine involved in multiple biological processes in diverse cell types (reviewed by Li et al., 2006). In the immune system, the central role of TGF-β is believed to be the maintenance of tolerance via the regulation of lymphocyte proliferation, differentiation, and survival. The differentiation state of target cells and the presence of additional regulatory signals, including co-stimulatory molecules and

inflammatory cytokines, influence TGF-β regulation of T cell activity (Cerwenka and Swain, 1999; Kehrl et al., 1986). In consequence, depending on the context TGF-β can induce distinct outcomes on the immune response and even play opposing roles. Accordingly, under some circumstances, TGF-β have a pro-inflammatory action by promoting monocyte maturation into DCs (Geissmann et al., 1998; Jaksits et al., 1999; Strobl et al., 1996; Zhang et al., 1999) or by recruiting monocytes to the site of injury or inflammation via multiple mechanisms: it can act as a chemoattractant for monocytes (Wahl et al., 1987); it induces adhesion molecules, including LFA-1 and the fibronectin receptor on monocytes (Bauvois et al., 1992; Wahl et al., 1993), enabling their attachment to extracellular matrix; and it induces matrix metalloproteinases, which can dissolve vascular membranes and facilitate monocyte transmigration (Wahl et al., 1993). In addition, TGF-β can induce the expression of pro-inflammatory molecules on monocytes, like IL-1 and IL-6, thus potentiating inflammation (Turner et al., 1990; Wahl et al., 1987). On the other hand, under different conditions TGF-β might also have an anti-inflammatory action by preventing the development of T_H2 cells through inhibition of expression of GATA-3 (Gorelik et al., 2000; Heath et al., 2000). Interestingly, despite TGF-β inhibits naïve T cell proliferation, it has minimal effect on activated T cells (Cottrez and Groux, 2001). It is possible that TGF-β inhibition of naïve T cell activation in the absence of co-stimulation attenuates T cell responses to self-antigens under steady state, whereas reversal of TGF-β inhibition by strong co-stimulatory signals, which are often associated with infection, limits the suppressive activity of TGF-β during a normal immune response (Li et al., 2006).

In addition to proliferation, TGF-β can also inhibit $CD8^+$ T cell differentiation by preventing the expression of multiple effector molecules of CTLs. Early studies showed that $CD8^+$ T cells activated in the presence of TGF-β do not acquire typical CTL functions, which is likely due to TGF-β inhibition of perforin expression in activated $CD8^+$ T cells (Ranges et al., 1987). TGF-β inhibition of perforin expression was observed upon activation of resting T cells, implying perforin as a direct TGF-β target (Smyth et al., 1991). Co-stimulation through 4-1BB, a TNFR family member co-stimulatory receptor, was shown to reverse TGF-β inhibition of CTL differentiation, which is further enhanced by IL-12 but reduced by IL-4 (Kim et al., 2005). Therefore, TGF-β inhibition of $CD8^+$ T cell differentiation is modulated

by co-stimulatory receptor and cytokine signaling pathways. TGF-β also inhibits FasL expression in T cell lines (Genestier et al., 1999), thereby affecting the death receptor cytotoxic pathway of $CD8^+$ T lymphocytes. Further, TGF-β greatly attenuates IFN-γ expression in $CD8^+$ and also in T_H1 $CD4^+$ T lymphocytes, which relates with reduced T-bet expression (Ahmadzadeh and Rosenberg, 2005; Bonig et al., 1999). More recently, studies in the mouse suggested inhibitory effect of TGF-β on the expression of perforin, granzyme A and B, FasL and IFN-γ by $CD8^+$ T lymphocytes occurs at the transcription level (Thomas and Massague, 2005).

In the immune system, TGF-β can be produced by all the lymphoid cells and also by DCs, macrophages, mast cells and granulocytes. The function of TGF-β is mediated through a membrane receptor that can be formed by two or three subunits named TGF-β receptor (TGF-βR) 1, 2 and 3 (Li et al., 2006). TGFβ-R2 can bind free ligand, whereas TGFβ-R1 can only recognize ligand that is already bound with TGFβ-R2 and formation of a ligand-induced complex involving both TGFβ-R1 and TGFβ-R2 is required for signaling. In contrast, TGFβ-R3 lacks a cytoplasmic domain and appears to function mainly in the concentration and presentation of TGF-β to TGFβ-R1 and TGFβ-R2. Signaling by these receptors occurs via Smad-dependent and Smad-independent pathways (for a review, see Massague, 1998). Figure 8 summarizes the main outcomes of TGF-β signaling implicated on the regulation of T-cell responses.

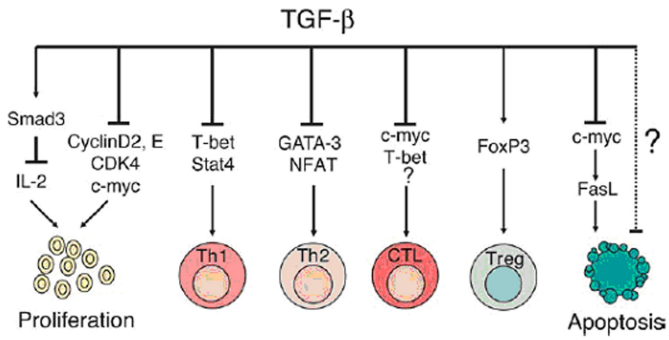

Figure 8. TGF-β regulation of immune responses. Adapted from (Li et al., 2006).

2.3.2. Chemokines: a matter of attraction

Chemokines are small and generally secreted molecules that play a major role in cellular trafficking and orchestration of lymphoid tissue architecture. Once released, they tend to remain concentrated locally, forming stable gradients that

guide the cells expressing the correspondent receptors throughout these gradients. Thanks to this mechanism, it is possible that rare antigen-specific T and B lymphocytes encounter antigen, as well as several distinct types of cells interact with each other. Leukocyte traffic therefore represents a key element in the regulation of the immune response. By displaying a different panel of chemokine receptors at the surface, the cells gain selective access to different types of tissues. Hence, the selectivity and flexibility necessary to regulate cell traffic under homeostatic and inflammatory conditions is provided by a differential tissue distribution of chemokines and a regulated expression of chemokine receptors on different leukocyte subsets (Sallusto and Lanzavecchia, 2000).

Chemokines and chemokine receptors are involved in the two distinct steps of leukocyte migration. The first is extravasation from the blood into lymph nodes, Peyer's patches, and inflamed tissues, a phenomenon also dependent on selectins and integrins. The second step controlled by chemokines and chemokine receptors is the migration to and positioning of leukocytes within secondary lymphoid organs and tissues (Sallusto et al., 2000).

Chemokines are classified into "lymphoid" and "inflammatory" based on the nature of the stimuli that induce their production and the site where it occurs (Sallusto et al., 2000). Lymphoid chemokines are produced within lymphoid tissues and are involved in maintaining homeostatic leukocyte traffic and cell compartmentalization within these organs. Inflammatory chemokines are produced by several cell types, including endothelial, epithelial, stromal and leukocytes, in response to a spectrum of inflammatory stimuli such as LPS, IL-1, and TNF-a. RANTES (acronym for regulated upon activation, normally T-expressed, and presumably secreted) and macrophage inflammatory proteins (MIP)-1 α and β are included in this subset.

In accordance to their differential functions in the immune system, T_H1 and T_H2 $CD4^+$ T cells express a different panel of chemokine receptors. Lymphocytes that have differentiated towards a T_H1 phenotype express preferentially CCR5, CXCR3, and CCR1, whereas in T_H2 cells CCR3, CCR4, CCR8 and an orphan chemoattractant receptor, CRT_H2 are the main chemokine receptors. CCR5 and CXCR3 are expressed also at lower levels on T_H2 cells (Sallusto et al., 2000).

Similarly to the previously described T_H1/ T_H2 feedback regulation, the cytokines produced by T_H1 cells, namely IFN-γ, up-regulate T_H1-attracting chemokines, such as RANTES, and antagonize T_H2-attracting chemokines.

Conversely, IL-4 and IL-13 produced by T_H2 cells stimulate the expression of T_H2-chemokines, like eotaxin, and down-modulate the expression of chemokines induced by T_H1 cells. TNF-α, a cytokine associated with both type 1 and type 2 responses, co-stimulates production of both type 1 and type 2 chemokines.

In respect to $CD8^+$ T lymphocytes, expression of CXCR3, CCR4, CCR5 and CCR7 can be found in different subsets. The later receptor is particularly important for T cells to gain access to secondary lymphoid tissues and is expressed by naïve and a subpopulation of memory cells known as "central memory" (discussed below). Conversely, CCR5 confers to lymphocytes the ability to migrate for the peripheral inflamed tissues and its expression is up-regulated following activation (for a review, see Sallusto et al., 2000) A recent work reports a further function for CCR5 in enabling naïve $CD8^+$ T cells to be directed to the sites where activated $CD4^+$ T cells interact with professional APCs (Castellino et al., 2006). Upregulation of CCR5 constitutes therefore a pivotal step in the orchestration of the lymphocyte interactions, facilitating the encounter of CD8 with activated $CD4^+$ T cells capable of delivering the appropriate helper signals necessary for the generation of memory $CD8^+$ T lymphocytes.

RANTES, MIP-1α and MIP-1β

The chemokine RANTES is a potent chemoattractant and activator of several classes of leukocytes, including T cells. The principal roles of this inflammatory chemokine, inferred from early *in vitro* studies, are exerting selective chemoattractant effects on monocytes and T cells (especially from the $CD45R0^+$ subset) and induction of transient changes in intracellular free calcium concentration in granulocytes and T cells (Kuna et al., 1992; Rot et al., 1992; Schall et al., 1990). In particular, RANTES induced calcium mobilization in T cells is biphasic, the initial, transient peak being predominantly associated with chemotaxis and the second, sustained one being associated with a spectrum of cellular responses characteristic of TCR activation, such as calcium channel opening, IL-2 receptor expression, cytokine release and T-cell proliferation (Bacon et al., 1995). These effects are directly related with the concentration of RANTES (reviewed by Appay and Rowland-Jones, 2001). At nanomolar concentrations, RANTES interact with a specific receptor (CCR1, CCR3, CCR4 or CCR5) to induce a transient calcium influx, which results in receptor polarization and cell

migration; however, at micromolar concentrations RANTES forms multimeric self-aggregates that potentially crosslink multiple cell-surface signaling molecules, including CD3, which induced a prolonged calcium influx and leads to a generalized cell activation state. Whether high concentrations necessary for RANTES aggregation can be found *in vivo* is not known. In addition, RANTES promotes cell adhesion of T lymphocytes by up-regulating the expression of a variety of cell-surface adhesion molecules, such as CD44, CD50 and CD28 (Szabo et al., 1997). Finally, an important anti-viral effect is also attributed to RANTES, as several viruses have developed a panel of evasion mechanisms to escape its effects (Appay and Rowland-Jones, 2001). The function of RANTES on anti-viral immunity might be related with the fact that it co-localizes with perforin and granzymes in the cytolytic granules of antigen-specific cells, being released along with those cytotoxic mediators following activation with the antigen (Wagner et al., 1998).

Activated $CD8^+$ T lymphocytes expressing CD45R0 are apparently the main source of RANTES in the immune system, as well as MIP-1α (Conlon et al., 1995). RANTES expression in T cells might be partially induced by TCR engagement and in endothelial cells the combined effects of IFN-γ and TNF-α have an enhancer effect on RANTES production at the mRNA and protein level (Dairaghi et al., 1998; Marfaing-Koka et al., 1995). Interestingly, RANTES mRNA is up-regulated late during T-cell activation (between days 3 and 5) and its expression is maintained in terminally differentiated T-cell lines (Schall et al., 1988). In the mouse system, expression of RANTES by memory $CD8^+$ T cells was reported to be controlled at posttranscriptional level, by a TCR-dependent mechanism. High levels of fully matured transcripts coding for RANTES remain silenced in the cytoplasm and protein synthesis occurs only after TCR stimulation, independently of CD28 (Swanson et al., 2002).

MIP-1α and MIP-1β are two related chemokines mainly produced by activated macrophages, but also by other activated immunocompetent cells such as lymphocytes, neutrophils, DCs, mast cells and NK cells, and whose main function is to attract other proinflammatory cells to sites of inflammation. In addition, these chemokines, as well as RANTES, can potently inhibit the macrophage uptake of HIV-1 via CCR5 ligation (Cocchi et al., 1995; Horuk, 1999). Production of MIP-1α and MIP-1β is induced by various proinflammatory stimuli,

including LPS, viral infection, TNF-α, IFN-γ and others, and down-regulated anti-inflammatory signals (like IL-4 and IL-10, for instance). Binding of MIP-1α and MIP-1β to their receptors, CCR5 or CCR1, elicits wide range of target cell functions including chemotaxis, degranulation and phagocytosis. In addition, as discussed above, as ligands of CCR5 these chemokines are likely involved in the modulation of T_H differentiation (Andres et al., 2000; Maurer and von Stebut, 2004).

3. Secondary T-cell immune responses

$CD8^+$ T-cell responses to specific pathogens are composed of distinct phases with different time-scales. The first phase occurs for a time-scale of few weeks and corresponds basically to the primary response after exposure to the pathogen, in which antigen-specific clones rapidly expand (by < 4-5 log), originating a population of effector cells. Subsequently, there is a massive contraction (by < 1-2 log) of the effector population which is programmed and independent of the magnitude of the initial expansion, antigen dose or duration of infection (Badovinac et al., 2002). The second phase involves the maintenance for a long time-scale (many years) of the small pathogen-specific population that escapes the contraction episode in the absence of re-exposure to the pathogen. Because these cells might potentially account for the control of a further infection upon re-challenge with the same pathogen, they are commonly named “memory cells”. The third phase consists of an increase in the number of this type of cells after re-exposure to the pathogen, which then provides protection. Exactly how protection is achieved is an item not completely understood.

3.1 Memory T-cell responses are different from primary responses

Memory T lymphocytes are distinct from the naïve counterparts in several ways, including surface markers, tissue distribution, activation status, kinetics of proliferation and differentiation, and requirements for survival.

3.1.1. Memory cell subsets have particular turnover and activation properties

Antigen-experienced T lymphocytes can be subdivided in two major subsets, according to their rate of turnover *in vivo* and expression of activation markers.

The first T lymphocyte subset is characterized by a relatively slow turnover (Geginat et al., 2003; Zimmerman et al., 1996), the absence of activation markers

and a similar pattern of distribution amongst lymphoid tissues to naive T cells (Sallusto et al., 1999b). Such non-polarized antigen-experienced lymphocytes were termed “central memory” T cells (T_{CM}) and commonly express CCR7 and CD62L, which, likewise naïve T cells, enables their entry in the lymph nodes and Peyer’s Patches via high endothelial venules. Although dividing only sporadically, resting memory cells are less inert than the naïve lymphocytes because they express higher levels of RNA as compared to naïve cells, which suggests that many of these cells are in at the late G1 phase of cell cycle (Veiga-Fernandes et al., 2000). T_{CM} have also a higher sensitivity to antigenic stimulation than naïve cells, are less dependent on co-stimulation, and up-regulate CD40L to a greater extent. Following TCR triggering, T_{CM} produce mainly IL-2, but after proliferation they efficiently differentiate into effector cells that loose CCR7 expression and produce large amounts of IFN-γ or IL-4, in the case of $CD4^+$ T cells, or cytotoxic mediators, in the case of $CD8^+$ T cells (Sallusto et al., 2004).

Other type of memory cells differ from the former described subset due to a clearly more activated state which closely resemble effector cells. For this reason, these cells were named “effector memory” T cells (T_{EM}). This cell subset has a rapid turnover and is characterized by rapid effector function. Hence, $CD8^+$ T_{EM} carry large amounts of perforin, and both $CD4^+$ and $CD8^+$ T_{EM} cells produce effector cytokines within hours following antigenic stimulation (Sallusto et al., 2004; Sallusto et al., 1999b). Human T_{EM} are memory cells that have lost the constitutive expression of CCR7, are heterogeneous for CD62L expression and display characteristic sets of chemokine receptors and adhesion molecules that are required for homing to inflamed tissues (Sallusto et al., 1999b). Such activated memory cells are therefore found in the blood and spleen, but are largely excluded from lymph nodes due to the absence of lymph node homing receptors, CD62L and CCR7. Nevertheless, these cells do enter lymph nodes in small numbers via afferent lymphatic vessels (Yawalkar et al., 2000).

The relative proportions of T_{CM} and T_{EM} in the human blood vary within the $CD4^+$ and $CD8^+$ T-cell compartments: T_{CM} is predominant within the $CD4^+$ and T_{EM} within the $CD8^+$. The exact relationship between T_{CM} and T_{EM} cells in humans remains unclear. In particular, whether T_{CM} and T_{EM} subsets represent two distinct populations derived from different precursors during the primary immune response or, alternatively, T_{EM} differentiate from T_{CM} after the primary response, is still a matter of debate. The same expanded clones could be found in both T_{CM} and T_{EM}

subsets within the influenza-specific $CD8^+$ memory T-cell pool for a period up to nine months, indicating that the same clonotypes can be present in both subsets in a stable way (Baron et al., 2003). However, in another study, the distribution of EBV-specific $CD8^+$ T lymphocytes for lytic versus latent epitopes was shown to be different amongst T_{CM} and T_{EM} subsets and dependent on the stage (acute versus chronic) of infection (Hislop et al., 2002). Hence, lytic epitope responses were dominant in the acute phase of infection, while latent epitope specificities were barely detected, and both had a uniform T_{EM} phenotype. In contrast, in the chronic stage of infection, where lytic epitope specificities were less abundant but in general still detected, and latent epitope specific cells were more frequent as compared to acute phase of infection, latent epitope responses show a greater tendency to acquire a T_{CM} phenotype, while lytic epitope specific cells remain preferentially in the T_{EM} compartment and show a propensity to express CD45RA, a phenomenon that will be discussed in further detail later in this manuscript.

3.1.2. Memory cells provide quantitatively and qualitatively enhanced protection

After the primary immune response, a fraction of activated T lymphocytes persist as circulating memory cells, which are able to give a quantitatively enhanced and qualitatively different response upon secondary challenge. First of all, despite the dramatic decline of T-cell number during the contraction phase, the precursor frequency of antigen-specific memory cells is far higher than for naïve T lymphocytes (Ahmed and Gray, 1996; Lin et al., 2000; Whitmire and Ahmed, 2000). An increased precursor frequency of antigen-specific cells is one of the reasons why secondary immune responses are accelerated upon re-exposure to antigen, in relation to primary immune responses. However, the significantly improved efficiency of secondary immune responses is also due to other important properties inherent to memory cells. In the case of T_{CM} lymphocytes, these include a shorter lag time for entering cell cycle, synthesizing cytokines, differentiating into CTL, and migrating to nonlymphoid tissues as compared to naïve cells (Cho et al., 1999; Garcia et al., 1999; Iezzi et al., 2001; Kedl and Mescher, 1998; Rogers et al., 2000; Veiga-Fernandes et al., 2000). In addition, T_{CM} cells are also less dependent on co-stimulation than naive T cells. In contrast, T_{EM} cells exert

immediate effector function and cytokine synthesis *in vivo* without prior activation, following re-exposure with the specific antigen (Hogan et al., 2001; Lanzavecchia and Sallusto, 2000).

The functional properties of T_{CM} and T_{EM} subsets are, thus, coordinated with their migratory routes: T_{EM} cells are believed to patrol the nonlymphoid tissues, providing immediate protection upon encounter with antigen, whereas T_{CM} cells, residing mainly within the secondary lymphoid organs, are reactivated by antigen in the draining lymph nodes where they activate APCs and undergo extensive clonal expansion, generating new waves of effector cells before migrating to nonlymphoid tissues. Despite the lag time associated to the reactivation of T_{CM}, it is well established that both T_{CM} and T_{EM} are more sensitive than naïve cells to TCR stimulation and provide an improved immune response upon a second encounter with antigen (Lanzavecchia and Sallusto, 2000). In summary, due to their inherent differential functional properties, T_{CM} and T_{EM} subsets may play complementary protective roles on the immune response, depending on the nature of antigen and/or route of the challenge (Bachmann et al., 2005a; Bachmann et al., 2005b).

3.1.3. Memory cells have distinct survival requirements

In the absence of perturbation, the steady state number and diversity of T cells in the peripheral lymphoid compartment is controlled through generation, death, survival and turnover. These mechanisms are largely regulated through competition for extrinsic limiting resources, including contact with self peptide-MHC complexes and cytokines such as IL-7 and IL-15 (Grossman et al., 2004; Tanchot et al., 1997). While both naïve $CD4^+$ and $CD8^+$ T cells require interaction with self peptide-MHC, as well as IL-7 for survival, memory cells can be maintained in the apparent complete absence of specific antigen (Garcia et al., 1999; Tanchot et al., 1997; Veiga-Fernandes et al., 2000). Experiments of adoptive transfer of memory cells into $MHC^{-/-}$ hosts have shown that both $CD4^+$ and $CD8^+$ memory cells can survive for long periods without TCR stimulation (Hu et al., 2001; Murali-Krishna et al., 1999; Swain et al., 1999). Whether long-term survival in $MHC^{-/-}$ hosts applies to both T_{CM} and T_{EM} remains to be determined.

Although interaction with peptide-MHC is not vital for keeping memory cells alive, it is extremely important to maintain memory cell functional quality (Kassiotis et al., 2002). In addition, cytokines, in particular the pro-survival cytokines IL-7 and IL-15 constitutively produced by DCs and stromal cells in the lymphoid tissues, were shown to be very important in maintaining T-cell memory homeostasis (Goldrath et al., 2002; Ma et al., 2006).

3.1.3.1. IL-15

The specific role of IL-15 is to support proliferation of the $CD8^+$ memory T-cell population (Becker et al., 2002; Goldrath et al., 2002; Schluns et al., 2002; Tan et al., 2002). In accordance, CD122 (IL-2Rβ), a component of the receptor for IL-15 (and IL-2), is selectively expressed in cells with a memory phenotype. Treatment with anti-CD122 monoclonal antibody considerably reduces normal turnover of memory cells *in vivo*, presumably because of a blockade of T cell contact with IL-15 (Ku et al., 2000). In the absence of IL-15 signals, antigen-specific memory $CD8^+$ T cells gradually decrease (Becker et al., 2002). This effect can be compensated by IL-7, but in the absence of both cytokines memory $CD8^+$ T-cell homeostatic proliferation is completely abrogated (Becker et al., 2002). Nevertheless, naïve T cells also express the receptor for IL-7 and, thus, compete with memory $CD8^+$ T cells for this cytokine. As naïve T lymphocytes largely outnumber memory $CD8^+$ T cells, the compensatory effect that IL-7 might have for the absence of IL-15 is always extremely limited. Further, these evidences strongly suggest that regulation of the naive and memory pools is interdependent and the size of each pool probably influences the homeostasis of the other (Jameson, 2002).

3.1.3.2. IL-7

As mentioned above, multiple lines of evidence indicate that signals delivered by IL-7 are important for maintaining memory $CD8^+$ T cells. The receptor for IL-7 (IL-7R) is composed of two chains: the IL-7Rα chain (CD127) and the common cytokine γ chain (γc, CD132), which is also part of the receptor for IL-2 and IL-15 (Ma et al., 2006). The α chain of the receptor for IL-7, which is uniformly expressed by naïve cells, is down-regulated following activation but is further re-

expressed on a subset of activated $CD8^+$ T cells that progress to become memory $CD8^+$ T cells. These evidences suggest that IL-7 may transmit the same survival signals to memory $CD8^+$ T cells as it does to naive cells (Goldrath et al., 2002; Schluns et al., 2000). Consistent with this idea is the finding that memory cells survive poorly after adoptive transfer into IL-$7^{-/-}$ recipient hosts (Schluns et al., 2000). A significant finding that might provide an explanation for this phenomenon was that IL-7 binding to its receptor increases expression of genes that promote lymphocyte survival and reduces expression of genes that induce lymphocyte apoptosis and cell cycle arrest (Khaled and Durum, 2003; Rathmell et al., 2001). Furthermore, expression of IL-7Rα parallels Bcl-2 expression, which is up-regulated upon treatment with IL-7. Therefore, IL-7 and Bcl-2 apparently work in concert to regulate the survival of resting T cells (Akashi et al., 1997; Maraskovsky et al., 1997).

In the case of memory $CD4^+$ T cells, cytokine dependence is more controversial since some evidences indicate that memory $CD4^+$ T cells are capable of homeostatic proliferation and antigen responses in the absence of signaling through γc receptors (Lantz et al., 2000; Tan et al., 2002), while other studies show that memory $CD4^+$ T cells require IL-7 signals in order to maintain basal proliferation and survival (Kondrack et al., 2003; Lenz et al., 2004; Seddon et al., 2003). Interestingly, in contrast to the persistent $CD8^+$ memory T-cell pool, the number of antigen-specific $CD4^+$ memory T cells declines steadily over time, suggesting that $CD4^+$ memory T cells might not be actively maintained (Homann et al., 2001). Therefore, identifying the factors that regulate $CD4^+$ memory T-cell homeostasis requires further investigation.

Interestingly, IL-7 binding to its receptor transiently down-regulates IL-7Rα on resting T cells (Park et al., 2004). This observation suggests the intervention of a feedback control mechanism in which T cells that have recently received an IL-7 signal, by down-regulating expression of the receptor, will not compete with those that have yet to encounter IL-7. In this way, size of the peripheral T-cell compartment would be maintained, with greater chances for survival of each T-cell clone.

3.1.3.3. IL-2

Unlike IL-15 and IL-7, IL-2 has been reported to have an inhibitory role on memory $CD8^+$ T-cell turnover (Dai et al., 2000; Ku et al., 2000). Since IL-2 is thought to play a supportive role in the activation of T cells (reviewed by Ma et al., 2006), the finding that it might also have a restrictive effect on lymphocyte proliferation is intriguing. One possibility is that IL-2 stimulates a suppressive population of IL-2-dependent regulatory $CD4^+$ T cells (Annacker et al., 2000; Sakaguchi, 2000; Suzuki et al., 1999). Hence, the balance in the relative concentrations of IL-15 and IL-2 could represent a key mechanism for controlling memory $CD8^+$ T-cell homeostasis.

3.1.4. Cell surface molecules associated to memory phenotypes

Antigen-experienced T cells express a spectrum of cell-surface molecules related to several functions, such as cell adhesion, migration, co-stimulation, survival, and others. Some of these surface markers are common to unprimed T lymphocytes, e.g., expression of CCR7 or CD27, and therefore can not be used to identify any specific subset per se (Hamann et al., 1997; Sallusto et al., 1999b). In contrast, low levels of CD62L or expression of CD45R0 in humans are exclusive of primed cells (Sprent and Surh, 2002). In addition, association of the expression profiles of several markers can be used to identify distinct sub-populations of antigen-experienced cells, such as the so called “central memory” ($CCR7^+CD45RA^-$) and “effector memory” ($CCR7^-CD45RA^-$) cells (Sallusto et al., 1999b).

3.1.4.1. CD45RA

A cell-surface molecule widely used to distinguish human naïve and primed T lymphocyte subsets is the leukocyte universal integrin CD45. In human cord blood almost all T cells express the CD45RA isoform, whereas cells expressing CD45R0 arise and accumulate progressively along life time of the individuals, reaching generally 40–60% of the total repertoire in the adult (Hayward et al., 1989). *In vitro* activation of $CD45RA^+$ T cells induces up-regulation of CD45R0 with concomitant loss of expression of CD45RA (Akbar et al., 1988). In addition,

the switching for CD45R0 phenotype is coincident with the acquisition of the ability to provide help for antibody production by B cells and, further, when compared with the $CD45RA^+$ counterparts, $CD45R0^+$ T cells seem to have a lower threshold of activation (Byrne et al., 1988; Clement et al., 1988). Collectively, these evidences led to the assumption that the $CD45RA^+$ population corresponds to un-primed, naïve cells, while memory and/or effector cells are comprised within the $CD45R0^+$ T-cell compartment (Dutton et al., 1998). If for $CD4^+$ T cells there are no evidences to date arguing against this presumption, it became clear that for $CD8^+$ T lymphocytes it was an oversimplification. Actually, nearly all $CD4^+$ and $CD8^+$ single-positive T cells from the cord blood and thymus express low levels of the integrin LFA-1 (Okumura et al., 1993). This lymphocyte ubiquitous cell-surface antigen, consisting of a heterodimer between the CD11a and CD18 molecules, plays an important role on binding to antigen presenting cells and is significantly up-regulated following lymphocyte activation. In the $CD4^+$ T cells of the adult peripheral blood, a high level of LFA-1 is found uniquely in the $CD45R0^+$ subset, as $CD45RA^+$ T-cells are homogeneously LFA-1^{low}. In contrast, despite the circulating $CD45R0^+$ $CD8^+$ T cells are also LFA-1^{high}, the $CD45RA^+$ compartment has a subpopulation that expresses high levels of LFA-1, in addition to the LFA-1^{low} subset (Okumura et al., 1993).

With the emergence of the tetrameric MHC-peptide complex technology, identification of antigen-specific T lymphocytes became possible. Studies using MHC tetramers associated with immunogenic peptides from human infecting viruses, such as EBV, HIV and CMV, showed that the viral-specific CD8-T cell clones are distributed amongst the $CD45RA^+$ and $CD45R0^+$ LFA-1^{high} subsets (Callan et al., 1998; Faint et al., 2001; Wills et al., 1999). The $CD45RA^+$ LFA-1^{high}

$CD8^+$ T subpopulation therefore comprises antigen-experienced cells. This conclusion was corroborated by the intracellular staining of these cells for effector molecules like perforin and IFN-γ (up to 85% and 93% of cells, respectively, were positive for those markers), as well as by the profile of expression of other cell-surface activation-related molecules, such as loss of CD62L and expression of CD57 (Faint et al., 2001; Vargas et al., 2001). In conclusion, expression of the CD45RA isoform can accurately be used to identify human naïve $CD4^+$ T lymphocytes, but for $CD8^+$ T cells CD45RA does not absolutely discriminate between naïve and antigen-experienced cells. Instead, an additional activation

marker is required, such as LFA-1, in order to differentiate the naïve (LFA-1^{low}) from the antigen-experienced (LFA-1^{high}) CD45RA-expressing cells.

Some markers used to define memory cells were reported to be partially reversible on late memory cells (Walker et al., 1995; Wills et al., 1999; Zimmerman et al., 1996). This is the case, for instance, of CD62L and CD45RA, which are expressed at very low levels in some subsets of antigen-experienced cells, but whose expression can be found in other primed lymphocyte subsets. Nevertheless, it is unclear whether this reversion is real, or reflects preferential survival of cells that failed to lose these markers during the primary responses (Pihlgren et al., 1996; Wills et al., 1999). This ambiguity also applies to CCR7 expression in human central memory population (Sallusto et al., 1999b), since up to date it has not yet been clearly demonstrated if this population can directly result from activation of naïve T cells, where CCR7 expression is never lost or, instead, derives from effector memory cells that have reacquired CCR7 expression, or both.

Finally, other cell-surface molecules, such as CD28 and CD27, are thought to be irreversibly lost during the differentiation process in which initial naïve T-cell precursors are converted into fully mature effector and memory T cells. These phenomena will be discussed in more detail later in this manuscript.

3.1.4.2. CD28

In the adult human peripheral blood it can be found a $CD8^+$ T-cell population that have lost surface expression of CD28. This population expands under conditions of chronic activation of the immune system, such as viral infections, autoimmune diseases, bone marrow transplantation or tumors, and also in the normal process of ageing. $CD8^+$ T lymphocytes specific for viral peptides of EBV, HIV and CMV were detected within the $CD28^-$ compartment and cells with this phenotype express perforin at considerable levels (Appay et al., 2002; Borthwick et al., 2000; Posnett et al., 1999). $CD28^-$ $CD8^+$ T cells are nevertheless absent from the thymus and only very low frequencies have been detected in cord blood. Therefore, these cells clearly constitute an antigen-experienced subset. In accordance, they are morphologically large, granular lymphocytes that express, amongst other cell-surface activation associated molecules, CD57 and high levels of LFA-1 (Azuma et al., 1993). In addition, $CD28^-$ $CD8^+$ T cells produce high levels

of IFN-γ *in vivo* and mediate potent cytolytic activity *in vitro*, but display poor proliferative capacity and significantly shorter telomeres when compared to the $CD28^+$ subset, which suggests a history of increased cell divisions (Batliwalla et al., 2000). Long-lived TCR oligoclonal V_β expansions can also be found *in vivo* within the $CD28^-$ population. Furthermore, evidences from *in vitro* studies indicate that firstly, $CD28^+$ $CD8^+$ T cells relatively prone to apoptosis, which is consistent with the high proliferation rate observed in this population; secondly, $CD28^-$ $CD8^+$ T cells originate from $CD28^+$ $CD8^+$ precursor cells; and lastly, $CD28^-$ $CD8^+$ T cells are relatively resistant to apoptosis when compared with their $CD28^+$ $CD8^+$ precursors. Therefore, $CD8^+$ T lymphocytes that escape apoptosis might mature into $CD28^-$ $CD8^+$ perforin-expressing effector cells (Posnett et al., 1999). Finally, several factors were shown to influence the loss of CD28 on $CD8^+$ T lymphocytes *in vitro*, such as proliferation and the presence of type I interferons (IFN-α and IFN-β), a type of cytokines that are produced during viral infections and indirectly promote the clonal expansion of CTLs *in vivo* (Borthwick et al., 2000). Taken altogether, these evidences suggest that $CD28^-$ $CD8^+$ T lymphocytes are terminal differentiated cells that have evolved by several rounds of division from $CD28^+$ $CD8^+$ T-cell precursors.

3.1.4.3. CD27

As described previously, CD27 has a constitutive expression on NK cells, antigen experienced B cells and naïve $CD4^+$ and $CD8^+$ T cells (reviewed by Croft, 2003; Watts, 2005). CD27 is transiently upregulated after T-cell activation *in vitro*, the peak expression level occurring between 24 and 72 hours, which suggests CD27 is important early in the response (de Jong et al., 1991; Gravestein et al., 1993). Furthermore, activation induces the production of the soluble form of CD27, which can be found in the supernatant of activated lymphocyte cultures and in the body fluids of healthy individuals as well (Hintzen et al., 1991b; Hol et al., 1993). A recent report describes that surface expression of CD27 can also be transiently downregulated by days 5 to 9 after stimulation in cultures of viral-specific human $CD8^+$ T-cell clones (Ochsenbein et al., 2004), but *in vivo* evidence of such phenomenon was never found. Later in the response, expression of CD27 can be irreversibly lost, likely after $CD8^+$ T cells had undergone several rounds of division (Hamann et al., 1999; Rufer et al., 2003; Ochsenbein et al., 2004). Conversely, in

$CD4^+$ T-cells CD27 expression can apparently be down-regulated without substantial cellular division (Baars et al., 1995).

Adoptive transfer of an autologous HIV-specific $CD8^+$ T-cell clone expanded *in vitro* and followed *in vivo* for more than 3 months revealed a propensity for $CD27^-$ cells to disappear, while $CD27^+$ infused cells persisted at large numbers at day 104 post-transfer. Since a stable reversion from a $CD27^-$ to a $CD27^+$ state was never observed *in vitro*, and because $CD27^+$ cells exhibit increased proliferation and resistance to apoptosis *in vitro* than $CD27^-$ cells, it was suggested that CD27 expression could confer a survival advantage to T cells *in vivo* (Ochsenbein et al., 2004). However, it can not be excluded that the clinical settings generated by HIV infection favor the accumulation of $CD27^+$ cells, and a distinct situation (i.e., persistence of $CD27^-$ cells) could occur in a different type of infection, such as infection with human cytomegalovirus (CMV). Actually, downregulation of CD27 on $CD8^+$ T-cells correlates with the acquisition of effector functions, such as the expression of perforin, granzyme B and FASL (Hamann et al., 1997). Interestingly, in the peripheral blood, the highest relative frequency of cells specific for CMV displays a $CD27^-CD8^+$ phenotype. Conversely, this population is markedly decreased in HIV infection (Appay et al., 2002; Kuijpers et al., 2003), which raised the possibility that the failure in the control of this viral infection might be due to the lack of $CD27^-CD8^+$ specific cells (Champagne et al., 2001; van Baarle et al., 2002a; van Baarle et al., 2002b). Also in the mouse model one can observe the loss of CD27 expression; effector-memory LCMV-specific $CD8^+$ T cells have a $CD27^{low/int}$ phenotype, whereas the central memory population is $CD27^+$ (Wherry et al., 2003). In addition, CD27 downregulation is claimed to be an irreversible event on both $CD4^+$ and $CD8^+$ T cells (De Jong et al., 1992; Hintzen et al., 1993; Ochsenbein et al., 2004). Altogether, the prevailing data suggest $CD27^-CD8^+$ population seems to result from the continuous stimulation of $CD27^+$ precursors and presumably represents a more differentiated antigen-experienced owing a more extensive replicative history and more complete effector functions that $CD27^+CD8^+$ cells.

3.2 Immunological memory generation and lineage relationships

Following primary immune responses, a small proportion of T cells escape survive the wide-scale elimination of effector T cells and become long-lived

memory cells. How these long-lasting memory cells generated and selected during the primary immune response is an issue difficult to address directly. However, several lines of evidence allowed the envisagement of a model to explain T-cell memory generation in humans (Sallusto et al., 2004; Sprent and Tough, 2001). According to this model, memory formation is a rather complex mechanism largely dependent upon multiple factors, such as the density of antigen, TCR affinity, local contact with particular cytokines and the nature of the APC. As discussed above, these factors establish the strength of stimulation that lymphocytes receive and determine their fate. Hierarchical activation thresholds are thought to progressively drive naïve T cells to proliferate, acquire fitness, differentiate and ultimately die by AICD (Figure 9a). As cytokine stimulation and encounter of T cells with APCs are probabilistic events that vary throughout the immune response, lymphocytes participating in the same response will receive different degrees of stimulation. This can lead to the arrest of primed T cells in different stages of the activation pathway, in addition to lymphocytes that have reached fully differentiation. In the early stages of the immune response, rapid replication of the pathogen ensures continuous entry of large numbers of activated APCs into the T-cell zones of the secondary lymphoid organs. Consequently, there is a huge density of APCs carrying high doses of antigen and co-stimulatory molecules, and secreting large amounts of cytokines. T cells can thus rapidly accumulate signals that enable them to become fit, proliferate and be converted into fully differentiated effector cells. At later stages of the response, elimination of the pathogen at the site of infection by effector cells reduces inflow of antigen-laden APC into the T-cell zones. Under these conditions, T-cell interaction with diminishing numbers of antigen-bearing APCs exhausts the capacity of APCs to produce stimulatory cytokines (Langenkamp et al., 2000). The exhausted APCs continue to elicit T-cell proliferation but cytokine production by T cells and formation of fully differentiated effector cells are reduced. Therefore, polarized cells would develop as the result of prolonged exposure to antigen, whereas non-polarized cells would arise by default when antigen is limiting (Iezzi et al., 2001; Langenkamp et al., 2002). This spectrum can be simply resolved into distinct subsets of T_{CM} and T_{EM} surviving after antigen clearance (Sallusto et al., 2004). Taken together, this model for T lymphocyte activation, generally known as «progressive differentiation model», proposes that generation of effector cells would be favored in early time points, whereas non-effector cells, that could constitute the precursors of fit, long lived-

memory cells, would be preferentially generated at later stages of the immune response (Gett et al., 2003; Lanzavecchia and Sallusto, 2002; Malherbe et al., 2004).

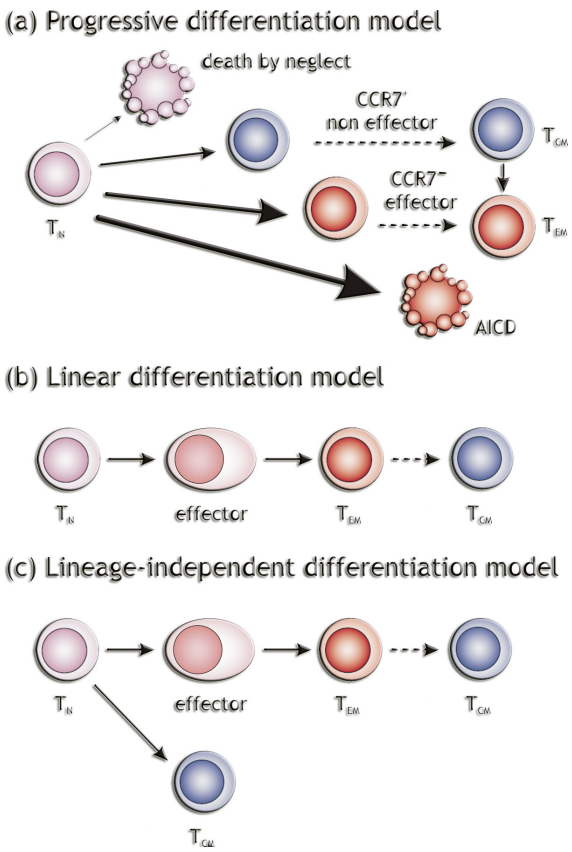

Figure 9. Models of memory T-cell differentiation. (a) The progressive differentiation model assumes that T-cell differentiation depends on the strength of stimulation and it is the most consensual model for human T-cell differentiation. The duration and intensity of antigenic stimulation is depicted by the length and thickness of the solid arrows. (b) Deduced from the mouse model, the linear differentiation model proposes a pathway whereby memory T cells are direct descendants of effector cells and memory T-cell development occurs only after decline of antigen concentration. (c) A variation of the previous model, the lineage-independent model predicts that T_{CM} cells can also be generated independently of the effector-cell lineage. Dashed arrows represent antigen-independent events leading to T cell proliferation and differentiation. AICD, activation-induced cell death.

This model is supported by several observations, including that only non-polarized *in vitro* generated cells recirculate through secondary lymphoid tissues (Iezzi et al., 2001; Weninger et al., 2001) and that T_{EM} have a lower proliferative potential and enhanced effector functions (Champagne et al., 2001; Hislop et al., 2001; Masopust et al., 2001; Wherry et al., 2003).

The previously described model implies that continuous exposure to antigen induces exhaustive differentiation (death) of virtually all of the effector cells, whereas a less-protracted contact with antigen, in addition to an extensive proliferative episode and an eventual effector stage, would induce in memory T-cell precursors incomplete or reversible death pathways. This hypothesis is corroborated by several observations in which different doses of antigen elicited different cellular outcomes, such as live bacteria or antigen formulated with microbial products, which induce fully differentiated CTL and T_H1 cells; killed

bacteria or antigen associated with the bacterial cell wall–derived Ribi adjuvant, which induce expansion of non effector $CD8^+$ or $CD4^+$ T cells; or antigens cross-presented by immature DC, which induce abortive proliferation or expansion of non-effector cells depending on the level of antigen expression (for a review, see Sallusto et al., 2004). Therefore, the gradient of signal strength is associated with the fitness of T cells involved in the immune response: cells receiving signals of suboptimal strength would be unfit and die by neglect, whereas higher strengths of signal would drive memory precursors to up-regulate anti-apoptotic molecules and receptors for homeostatic cytokines, therefore becoming fit. At extreme conditions of high strength of signal T cells could eventually be deleted by activation induced cell death.

In the mouse, the dynamics and relationship between T_{CM} and T_{EM} have been analyzed within the $CD8^+$ T-cell compartment by adoptive transfer experiments and revealed surprising differences in relation to human memory cells (Bouneaud et al., 2005; Wherry et al., 2003). Interestingly, in the absence of antigen, T_{CM} are extremely stable in vivo, whereas T_{EM} are present only transiently. The data also indicates that T_{EM} have a high propensity to undergo apoptosis and die in few weeks when antigen is not present, with the exception of a small subset that is able to convert into T_{CM} by reacquiring CCR7 and CD62L expression (Bouneaud et al., 2005). A recent report has shown that the capacity of T_{EM} to acquire a T_{CM} phenotype strongly depends on the initial frequency of naïve T-cell precursors, the T_{EM} cells generated at low precursor frequency conditions constituting a stable lineage with limited capacity of conversion into T_{CM} (Marzo et al., 2005). In addition, the rate of conversion from T_{EM} to T_{CM} is inversely proportional to the strength of stimulation (Wherry et al., 2003). Finally, T_{CM} convert into T_{EM} upon antigenic re-stimulation, but not under steady-state conditions. These findings strongly suggest that in mice the level of antigen persistence shapes the composition of the memory $CD8^+$ T-cell pool and that T_{CM} and T_{EM} do not necessarily represent distinct subsets, but rather belong to a continuum in a linear naïve → effector → T_{EM} → T_{CM} differentiation pathway (Wherry et al., 2003 and Figure 9b). By contrast, in humans both T_{CM} and T_{EM} subsets are rather stable, without evidence of T_{EM} → T_{CM} inter-conversion at steady-sate (Baron et al., 2003). Furthermore, human T_{EM} also fail to reacquire stable CCR7 expression *in vitro* (Langenkamp et al., 2003). A repertoire study has also shown that there is a significant overlap of clones between T_{CM} and T_{EM}

repertoire in the H-Y mouse model, demonstrating that some T_{CM} and T_{EM} cells have arisen from a common naïve T cell-precursor (Bouneaud et al., 2005). This finding that T_{CM} and T_{EM} repertoires are partially distinct and therefore some T_{CM} and T_{EM} clones could arise independently, on a stochastic basis, based on pre-commitment of the individual naive T cell or depending on its stimulation conditions, such as cytokine environment or access to $CD4^+$ T-cell help (Figure 9c).

Collectively, these data strongly suggest that the mechanisms implicated in the generation and dynamics of memory T-cell populations are significantly different between mouse and man.

PART III. AIMS AND METHODOLOGICAL APPROCHES

Sallusto and Lanzavecchia have shown for the first time that expression of the lymph node homing receptor CCR7 can be used to separate, both $CD4^+$ and $CD8^+$ human $CD45R0^+$ T cells, into two functionally distinct subsets: $CCR7^+$ $CD45RA^-$ (T_{CM}) and $CCR7^-$ $CD45RA^-$ (T_{EM}). Unlike $CD4^+$ T cells, in the $CD8^+$ compartment it can be found an additional $CCR7^-$ subset that expresses CD45RA (T_{EMRA}). Confocal microscopy has revealed that this population harbor cells expressing the highest levels of perforin (Sallusto et al., 1999b). It was therefore suggested that the T_{EMRA} subset should correspond to a population of terminally differentiated $CD27^-$ effector cells, previously described by Hamann et al. (1997). These cells display a V_β repertoire significantly different from naïve cells, containing oligoclonal expansions of particular TCR V_β elements, and also have shorter telomeres, suggesting that cells of the $CD45RA^+CD27^-$ subset have been selected *in vivo* through antigen stimulation and evolved through extensive rounds of division (Hamann et al., 1999). The same authors had proposed that CD45RA and CD27 expression could be used to identify naïve ($CD45RA^+$ $CD27^+$), memory ($CD45RA^-$ $CD27^+$) and effector ($CD45RA^+$ $CD27^-$) $CD8^+$ T cells in humans (Hamann et al., 1997). This classification, however, underestimates the complexity of the memory $CD8^+$ T-cell subset revealed by the expression of CCR7. For instance, memory cells defined by the $CD45RA^-$ $CD27^+$ phenotype would therefore include both T_{CM} and T_{EM}, which were shown to enclose a distinct functional specialization (Champagne et al., 2001; Sallusto et al., 1999b). Whether memory $CD27^+$ $CD8^+$ T cells can additionally be harbored within the $CD45RA^+$ subset has also been questioned. $CD27^+$ $CD8^+$ T cells specific for several human viruses can be found within the $CD45RA^+$ compartment during the chronic phase of infection (Appay et al., 2002). In addition, heterogeneity of the effector/memory compartments was shown to be further extended to CD28 expression in a study where $CD8^+$ T cells specific for HIV, CMV, EBV and hepatitis C virus (HCV) were extensively characterized regarding their surface phenotype, intracellular functional molecules and *ex vivo* cytotoxic capacity. Based on these data, the authors showed that co-expression of CD27 and CD28 receptors could be used to distinguish three functionally different subsets of $CD8^+$ T cells according to the expression of effector functions: early ($CD27^+$ $CD28^+$, DP), intermediate ($CD27^+$

$CD28^-$, 27SP) and late ($CD27^-$ $CD28^-$, DN) differentiated cells (Appay et al., 2002). This classification reflects, thus, the activation status of antigen-experienced $CD8^+$ T cells, rather than discriminating effector and memory cells. In particular, CD27 and CD28 expression does not allow distinguishing T_{CM} from T_{EM}, nor T_{EM} from T_{EMRA}.

The prevailing data concerning the description of naïve, effector and memory $CD8^+$ T-cell populations in humans remains, thus, rather fragmentary. Manifestly, analyses of $CD8^+$ T cells including solely two or three parameters are not sufficient to reveal the whole heterogeneity of the antigen-experienced $CD8^+$ lymphoid compartment. The compound subsets are not clearly established, especially within the T_{EM} and T_{EMRA} compartments, and the correspondent differential roles and lineage relationships remain undisclosed. However, understanding the distribution, function and relationship of the $CD8^+$ T-cell subpopulations is of fundamental value for the monitoring of the immune system in several experimental and clinical situations.

The present study aims to elucidate some still unclear aspects concerning the heterogeneity of the human $CD8^+$ T-cell compartment. In particular, this study focuses on the distribution of circulating human $CD8^+$ T cell populations based on the simultaneous association of CCR7, CD45RA, CD27 and CD28 cell-surface markers. The discrete populations that have been identified in the total $CD8^+$ T-cell pool were further isolated by cell-sorting and gene expression of 18 genes was assessed, simultaneously, in single-cells by a novel multiplex RT-PCR method we developed. Our results indicate that the phenotype resulting from this combination of markers permits to discriminate seven types of functionally different populations: naïve, recently-activated cells, T_{CM}, T_{EM}-DP, T_{EM}-27SP, T_{EM}28SP and T_{EM}-DN. CD45RA expression is required to define the naïve subset, but does not discriminate functionally different populations of primed cells. Although the methods used here did not allow the categorization of the nature of cells that mediate immunological memory versus effectors, our data provide important evidence that help to establish the stages of differentiation of $CD8^+$ T cells from naïve cells to cytolytic effectors. In particular, our data suggest that the loss of surface receptors can occur asynchronously during the differentiation process and T_{EM} and T_{EMRA} subpopulations might play similar roles in the immune response *in vivo*. In summary, by crossing several markers that were independently used in previous studies, in association with single-cell multiplex gene expression

analysis, this work will help to clarify and consolidate some important features of the biology of $CD8^+$ T cell populations.

The results of this thesis are described in the following section by means of two scientific articles that approach, respectively, the development of a novel methodology of RT-PCR that allows the study of an extended number of functions expressed in single cells (published in Genome Research), and the characterization of the $CD8^+$ T-cell subsets found in the human blood by gene expression profiling (submitted for publication).

RESULTS

MANUSCRIPT #1

Quantification of Multiple Gene Expression in Individual Cells

António Peixoto, Marta Monteiro, Benedita Rocha and Henrique Veiga-Fernandes

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Quantitative gene expression analysis aims to define the gene expression patterns determining cell behavior. So far, these assessments can only be performed at the population level. Therefore, they determine the average gene expression within a population, overlooking possible cell-to-cell heterogeneity that could lead to different cell behaviors/cell fates. Understanding individual cell behavior requires multiple gene expression analyses of single cells, and may be fundamental for the understanding of all types of biological events and/or differentiation processes. We here describe a new reverse transcription-polymerase chain reaction (RT-PCR) approach allowing the simultaneous quantification of the expression of 20 genes in the same single cell. This method has broad application, in different species and any type of gene combination. RT efficiency is evaluated. Uniform and maximized amplification conditions for all genes are provided. Abundance relationships are maintained, allowing the precise quantification of the absolute number of mRNA molecules per cell, ranging from 2 to $1{,}28\times10^{9}$ for each individual gene. We evaluated the impact of this approach on functional genetic read-outs by studying an apparently homogeneous population (monoclonal T cells recovered 4 d after antigen stimulation), using either this method or conventional real-time RT-PCR. Single-cell studies revealed considerable cell-to-cell variation: All T cells did not express all individual genes. Gene coexpression patterns were very heterogeneous. mRNA copy numbers varied between different transcripts and in different cells. As a consequence, this single-cell assay introduces new and fundamental information regarding functional genomic read-outs. By comparison, we also show that conventional quantitative assays determining population averages supply insufficient information, and may even be highly misleading.

Genome Research (2004) 14:1938-1947

Quantification of Multiple Gene Expression in Individual Cells

António Peixoto, Marta Monteiro, Benedita Rocha,[1] and Henrique Veiga-Fernandes

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Quantitative gene expression analysis aims to define the gene expression patterns determining cell behavior. So far, these assessments can only be performed at the population level. Therefore, they determine the average gene expression within a population, overlooking possible cell-to-cell heterogeneity that could lead to different cell behaviors/cell fates. Understanding individual cell behavior requires multiple gene expression analyses of single cells, and may be fundamental for the understanding of all types of biological events and/or differentiation processes. We here describe a new reverse transcription-polymerase chain reaction (RT-PCR) approach allowing the simultaneous quantification of the expression of 20 genes in the same single cell. This method has broad application, in different species and any type of gene combination. RT efficiency is evaluated. Uniform and maximized amplification conditions for all genes are provided. Abundance relationships are maintained, allowing the precise quantification of the absolute number of mRNA molecules per cell, ranging from 2 to 1.28×10^9 for each individual gene. We evaluated the impact of this approach on functional genetic read-outs by studying an apparently homogeneous population (monoclonal T cells recovered 4 d after antigen stimulation), using either this method or conventional real-time RT-PCR. Single-cell studies revealed considerable cell-to-cell variation: All T cells did not express all individual genes. Gene coexpression patterns were very heterogeneous. mRNA copy numbers varied between different transcripts and in different cells. As a consequence, this single-cell assay introduces new and fundamental information regarding functional genomic read-outs. By comparison, we also show that conventional quantitative assays determining population averages supply insufficient information, and may even be highly misleading.

[Supplemental material is available online at www.genome.org.]

Functional genomic analysis is fundamental for understanding how genomic expression profiles influence cell fate. Such studies are usually performed by using either micro-arrays or a real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). These methodologies can determine multiple gene expression, but have a major limitation. They only allow studies at the population level and thus only determine average gene expression. They cannot evaluate variations of gene expression between individual cells. However, in many types of biological events, individual cells within apparently homogeneous populations have different fates. It is likely that these different fates are conditioned by different patterns of gene expression. Because the events occurring in each individual cell are unknown, current methods may fail to identify the gene expression balance that ultimately determines cell behavior. This latter information requires multiple gene expression analysis of single cells, which may be a fundamental step for the understanding of all types of biological events and/or differentiation processes.

Multiple analysis of gene expression at the single-cell level requires major technological advances. Most techniques are qualitative and only allow studies of the expression of a few genes (Phillips and Lipski 2000; Veiga-Fernandes et al. 2000; Walter et al. 2000; Lambolez et al. 2002). When more genes were to be tested, these methods were reported to have inherent biases (Phillips and Lipski 2000; Walter et al. 2000). Indeed, in more extensive gene expression studies, the efficiency of detection was simply not controlled (Ruano et al. 1995; Plant et al. 1997; Zawar et al. 1999; Gallopin et al. 2000).

In principle, there are no sensitivity limitations for single-cell gene expression analysis. Single-cell methods can detect genomic DNA, that is, two gene copies, when two successive PCR amplifications of the same gene are performed (Loffert et al. 1996). However, the modification of such methodology to allow multiple mRNA expression studies involves serious difficulties. First, the amount of mRNA extracted from a single cell is so minute that samples cannot be split. The expression of multiple genes must be investigated in the same sample and in the same RT-PCR round. This implies the presence of multiple primers and the generation of multiple amplicons in a single PCR round, which may induce serious competition between different amplifications. It was claimed that analysis of the coexpression of more than five genes in one cell simultaneously would necessarily lead to nonspecific inhibitions of amplification (Walter et al. 2000). These potential competition events may induce false-negative results that are particularly difficult to control in single-cell assays. Indeed, as each individual cell is potentially different, it is not possible to determine whether a negative result is due to the absence of gene expression or to the absence of amplification due to competition.

Further difficulties are involved in attempting to quantify gene expression in single cells. Such quantification would require the demonstration of the maintenance of abundance relationships between multiple genes and throughout multiple reactions: from mRNA to cDNA, and throughout two successive PCR amplifications. The template switching required by two-step amplifications may introduce potential bias (Phillips and Lipski 2000). Moreover, it was postulated that abundance relationships could not be maintained throughout exponential amplification, as theoretical mathematic analysis showed that hybridization kinetics during thermal cycling could induce both sequence- and copy number-dependent bias (Peccoud and Jacob 1996). However, certain techniques of enhanced reverse transcription faith-

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fully maintained relative abundance relationships using exponential amplification (Iscove et al. 2002; Makrigiorgos et al. 2002). These new findings open the possibility that RT-PCR methods could be modified in such a way that abundance relationships could still be maintained, allowing quantification of gene expression in individual cells.

Here we describe a new method in which all previous limitations have been overcome since the expression of 20 different genes can be quantified simultaneously in each cell. We further demonstrate that this powerful technique imparts fundamental new information on cell behavior. In contrast, we also show that gene expression studies performed at the population level do not impart sufficient information and may even be highly misleading.

RESULTS

General Aspects of Quantitative Single-Cell Multiplex RT-PCR

Sorted cells are lysed and the mRNA is retrotranscribed using specific 3′ primers. A first PCR follows, where both 3′ and 5′ primers for all 20 different genes are present in the same reaction (Fig. 1). The products of this first amplification are next split into individual wells where a second seminested real-time PCR amplifies each individual gene separately (Fig. 1). To quantify the number of mRNA copies of different genes, the cycle threshold (C_T) value obtained for each different gene product is then compared with a known quantified RNA standard that followed the same rules of retrotranscription and amplification of the tested samples. This comparison allows a precise determination of mRNA copy numbers of different genes from a single individual cell.

The feasibility of this method is strictly dependent on multiple parameters: a precise experimental strategy, which includes the use of specific reverse transcription (see Discussion), the use of precise rules for primer design, and particular amplification conditions (see Methods).

Validation of Primer Design Strategy

Efficiency

To allow comparison of the expression of different genes, PCR reactions amplifying different cDNA fragments must have the same efficiency. We tested the efficiency of our PCR amplifications on the cDNA from gut intraepithelial T lymphocytes (IELs), because this template contains all of the cDNAs coding for the 20 different genes we investigated. Aliquots of this template were amplified separately for each gene product, using primer combinations from either the first or the second PCR. The PCR accumulation slope on the linear phase of all of these PCRs (Fig. 2A) allowed us to evaluate PCR efficiency (Ramakers et al. 2003). We show that all 40 types of PCR had equal efficiency (Fig. 2B).

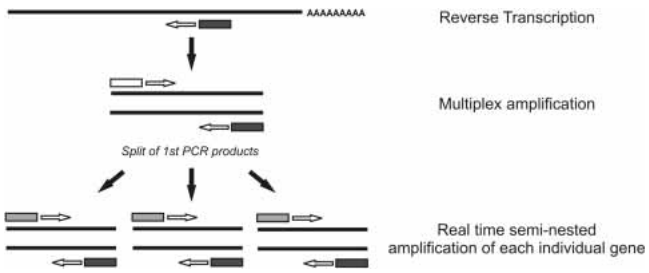

Figure 1 Outline of the quantitative multiplex single-cell RT-PCR. Single-cell mRNA is retrotranscribed using a 3′-specific primer for each individual gene of interest (dark gray box). Next, single-cell cDNA is amplified on a first multiplex PCR where both 3′ (dark gray box) and 5′ (white box) primers of all different genes are present (15 cycles). Products of the first amplification are next split for a second seminested real-time PCR where a nested 5′ primer (light gray box) and the 3′ primer (dark gray box) are used to amplify each gene separately. This second round of amplification allows a precise quantification because test samples are compared with an RNA standard submitted to the same RT and amplification protocol.

Competition

A key feature of our method is the first PCR reaction, where all 40 primers amplifying different cDNAs are present and the 20 cDNA types are amplified simultaneously. This is required for assessment of multiple gene expressions in the same cell. However, this multiple amplification may result in PCR inhibition and/or reduced PCR efficiency that may invalidate the data (Phillips and Lipski 2000; Walter et al. 2000). To evaluate competition, the same amount of IEL cDNA was amplified on the first PCR round: either separately for each individual gene, or in combination with all other genes. Next, the PCR products generated in these two conditions were amplified on a second quantitative PCR. As shown in Figure 2C,D, the relative levels of expression of each gene were the same when the gene was amplified separately or in combination.

We conclude that our method provides a variety of PCR amplifications with similar efficiencies. Moreover, the 20 primer combinations of the first PCR can be associated in the same reaction, showing that our methodology prevents competition between different amplifications, a major handicap of previous methods for multiple simultaneous amplifications (Phillips and Lipski 2000; Walter et al. 2000). Our strategy thus allows simultaneous multiple gene analysis in the same sample. Because all PCRs have the same efficiency, this method also allows the comparison of relative levels of expression of different genes between themselves.

Broad Spectra Application

We next investigated whether the strategy we used for this particular multiplex single-cell analysis could be applied for any other type of gene combination, and in different species. For that purpose we used the same rules described in the Methods section to select primers and amplicons that study multiple T-cell functions (including cytokine and chemokine expression) in human cells. We tested the efficiency of our PCR amplifications on cDNA from human small intestine, because this template also expresses all of the cDNAs we investigated. Aliquots of this template were amplified separately for each gene product, using primer combinations from either the first or the second PCR (Fig. 3A). Evaluation of PCR efficiency (Ramakers et al. 2003) revealed that all 40 of these individual PCR reactions had the same efficiency (Fig. 3B). To evaluate competition, the same amount of human small intestine cDNA was amplified on the first PCR round, either separately for each individual gene or in combination with all other genes. Next, the PCR products generated in these two conditions were amplified on a second quantitative PCR. As with the mouse multiple gene analysis (Fig. 2B,C), we found no inhibition due to primer/amplicon competition, as relative levels of expression were identical whether each gene was amplified separately or in combination (Fig. 3C,D).

We conclude that by using the primer/amplicon selection strategy and the amplification conditions described in the Methods section, this methodology can be applied to the simultaneous quantification of any 20 mRNA combinations. This validates the basic principles of the method for multiparameter analysis and provides proof of its broad spectrum of applications.

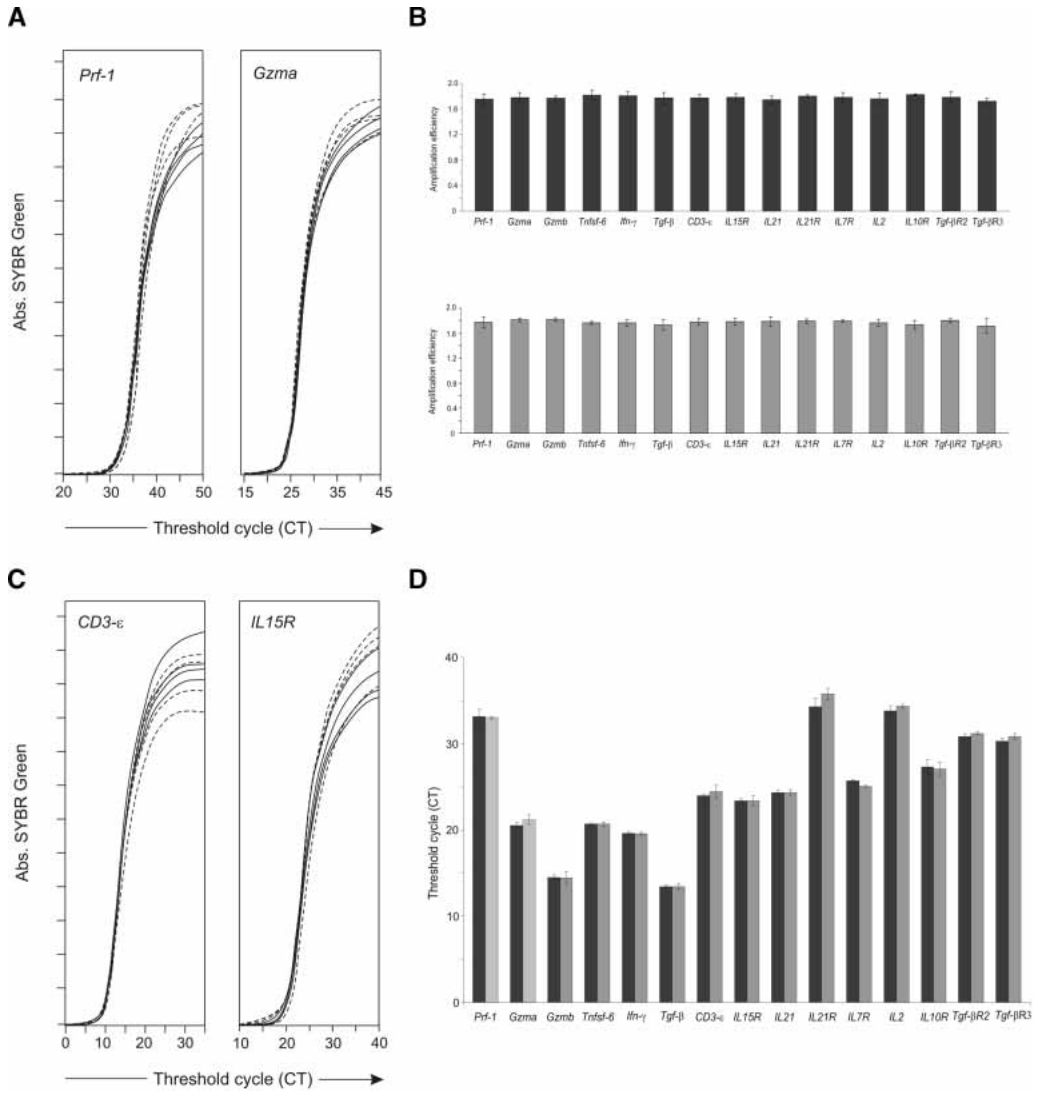

Figure 2 Efficiency and competition of PCR amplifications. (*A*,*B*) Aliquots of cDNA from mouse IEL were amplified separately for each gene and each type of PCR reaction to determine PCR efficiency. (*A*) Quadruplicate amplification slopes for *Prf-1*, *Gzma*. Set of primers from the first PCR (solid lines) the second PCR (dashed lines).The same tests were performed for all genes, giving the same results (*B*) Slope values from the quadruplicates were assessed on the exponential phase of the real-time amplification reaction, and PCR efficiencies were determined using LinRegPCR 7.0 software. Means ± SD of PCR efficiencies are shown for the first (*upper* panel) and second (*bottom* panel) PCRs. The significance of these differences was evaluated by ANOVA. Within each PCR, all primer combinations had the same efficiency. We also found no significant difference in the variance between the first and second PCR amplifications (ANOVA, $P > 0.1$). Data are from one of three independent experiments. (*C*,*D*) Competition: Aliquots of cDNA from IELs were amplified separately for each gene, or in multiplex in the first PCR round. Quadruplicates of these reactions were further amplified in a second real-time PCR. (*C*) Quadruplicates of amplification for *Cd3-ε*; *IL15r*, genes amplified in multiplex (solid line) or alone (dashed line). (*D*) Comparison of threshold cycle mean values (C_T) obtained for each different gene amplified in multiplex (black bars) or separately (gray bars). No significant differences were observed between the two amplification conditions for each different gene (*t*-test, $P > 0.1$).

From Population Studies to Single-Cell Studies

Template Switching

The quantification of the limited material recovered from single cells as well as the study of multiple parameters in a single cell requires the use of a two-step PCR amplification, and the consequent template switching from the first to the second PCR round. Such template switching may introduce potential bias (Phillips and Lipski 2000). To prevent such bias, the first amplification round should amplify rare templates in such a way that aliquot switching should exclude tube-to-tube variability. The initial amplification should also guarantee that highly expressed genes do not reach a saturation plateau that would exclude accurate quantitative assessments on the second PCR. In other words, to prevent biased assessments, the first round of amplification must preserve the initial representation of rare genes, simultaneously excluding excessive amplification of highly abundant gene copies.

We tested for bias introduced by the template switching between the first and second PCRs using several approaches. First, we studied the amplification of a synthesized double-stranded template, corresponding to the mouse *Gzma* sequence we amplified in our PCRs (Fig. 4A). Because the molecular weight of this template was known, we could calculate the absolute number of DNA molecules that was present in each reaction. In this way, we could study possible artifacts of two-step

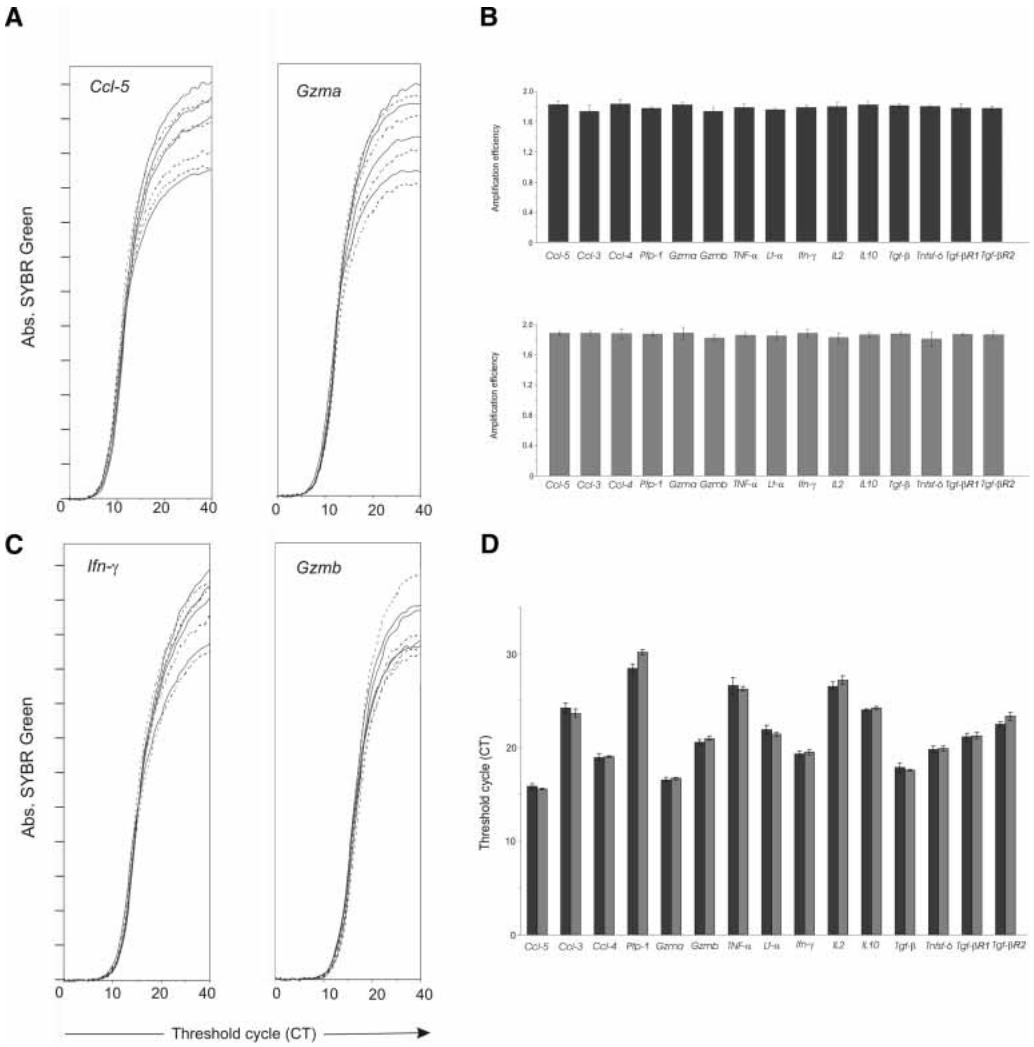

Figure 3 Broader applications for the quantitative single-cell multiplex RT-PCR. Aliquots of cDNA from human small intestine were tested as described in Figure 2. (*A*,*B*) Efficiency of PCR amplification for different gene products. Aliquots were amplified separately for each gene and each type of PCR. (*A*) Quadruplicate amplification slopes for *Ccl-5*, *Gzma*, first PCR (solid lines) and second PCR (dash lines). Amplification of all genes gave the same results (*B*) Slope values from the quadruplicates were assessed on the exponential phase; efficiency and significance of variation were performed as described in Figure 2. Results show mean ± SD of PCR efficiencies of the first (*upper* panel) and second (*bottom* panel) PCRs. All primer combinations had the same efficiency. We also found no significant difference in the variance between the first and second PCR amplifications (ANOVA, $P > 0.05$). Data are from one of three independent experiments. (*C*,*D*) Competition: Aliquots of cDNA from human small intestine were amplified separately for each gene, or in multiplex in the first PCR round. Quadruplicates of these reactions were further amplified in a second real-time PCR. (*C*) Quadruplicates of amplification for *Ifn-γ*, *Gzmb* genes amplified in multiplex (solid line) or alone (dashed line). Comparison of C_T values obtained for each different gene amplified in multiplex (black bars) or separately (gray bars). No significant differences were observed between the two amplification conditions for each different gene (*t*-test, $P > 0.1$).

reactions at both high and low copy numbers of starting material. Decreasing concentrations of this template, by a factor of 16, from 1.28×10^9 to four molecules, were amplified in single or double-step amplification (Fig. 4A). Upon a single amplification, the linear regression curve of this standard had a high correlation coefficient ($r^2 = 0.999$). These results show that we can assess a vast range of template copy numbers while maintaining linearity. Next, we studied the amplification of the same double-stranded template using a two-step amplification. The number of amplification cycles in the first PCR ranged from five to 30. When the first PCR was 15 cycles, high correlation coefficients ($r^2 = 0.999$) were maintained in the second PCR. Furthermore, using this 15-cycle preamplification, linearity was maintained at both high and low template concentrations (Fig. 4A). We tested these same parameters for other synthesized cDNA sequences, namely mouse *Gzmb* and *Prf-1* and human *CD3-ε*, and similar results were obtained (data not shown). We further investigated tube-to-tube variability at very low copy numbers directly. Triplicates of synthesized mouse *Gzma* sequence (four molecules) were amplified in a first 15-cycle PCR, and then aliquots of these preamplified products were amplified, using a quantitative PCR. We show that all samples had equal C_T values (Fig. 4B).

These results show that when a 15-cycle amplification is used in the first PCR, we can exclude the existence of bias or randomness introduced by template switching at template copy numbers from 1.28×10^9 to 4 molecules. It must be noted that at higher or lower amplification cycles on the first PCR, the second PCR does not follow the same rules of linearity (data not shown). When the first PCR has less than 15 cycles, low template

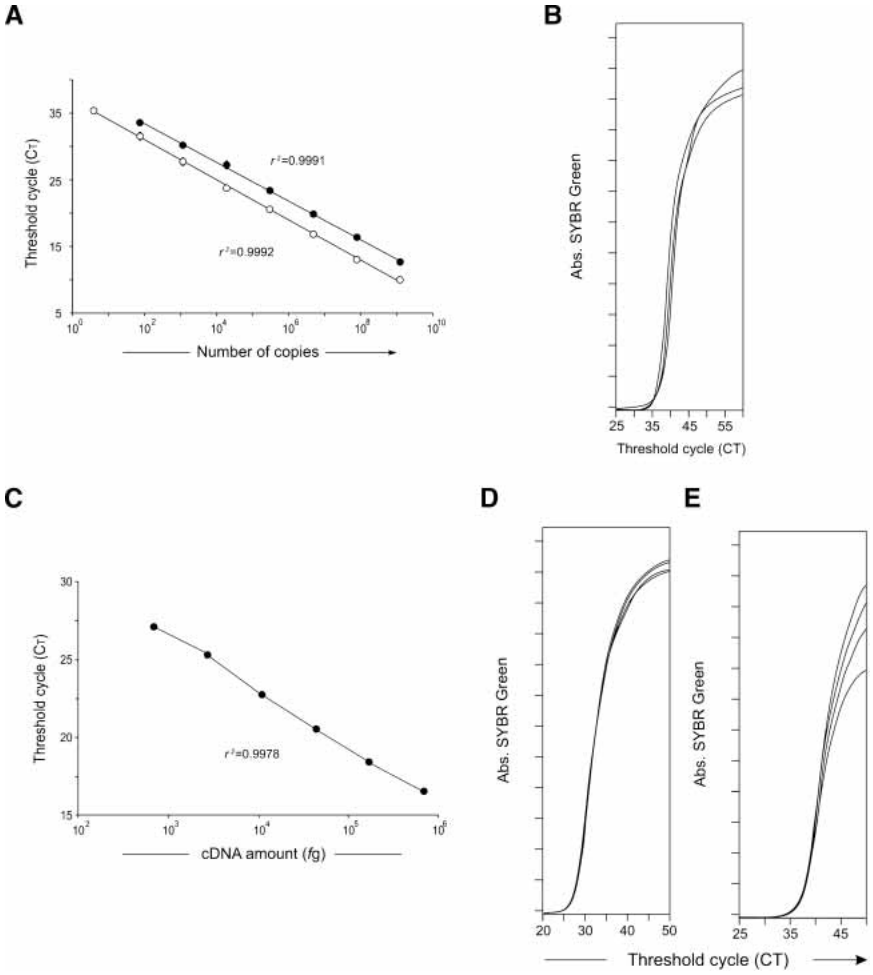

Figure 4 Linearity of double-round PCR amplification. (*A*) A double-stranded synthesized DNA sequence from *Gzma* was amplified in quadruplicate by a single PCR (●) or by a two-step PCR of 15 preamplification cycles (○). Decreasing template concentrations by a factor of 16 were used. Means ± SD of quadruplicates are included, although SDs frequently overlap with the symbols as we found little or no variation. Linear regression curves of these amplified standards are indicated and have similar high correlation coefficients (r^2 = 0.999). Similar results were obtained with three different types of synthesized cDNA. (*B*) Four molecules of a double-stranded synthesized *Gzma* sequence were amplified in triplicate using 15 cycles on the first PCR amplification followed by a second real-time PCR. PCR accumulation curves are shown. (*C*) A bulk cDNA population (7×10^5 to 680 *f*g) was used to amplify 28S (*Mrp-S21*) gene in quadruplicate by a two-step PCR of 15 preamplification cycles. Decreasing template concentrations by a factor of 4 were used. Mean values and standard deviations of quadruplicates are included, but SDs overlap with the symbols, as we found little or no variation. The linear regression curve of this amplified standard is indicated (r^2 = 0.9978). (*D*) Single cells were retrotranscribed and preamplified (15 cycles) for *Hprt-1*. A second quantitative PCR was used for the evaluation of the relative expression level (C_T) of *Hprt-1* on each individual cell. Quadruplicates of amplification are shown. (*E*) Single individual cells were sorted, and treated for genomic DNA amplification (see Methods). The *Gzma* gene was next amplified in a seminested PCR (15 cycles of preamplification). PCR accumulation curves are shown for four individual cells. Data are from one of three independent experiments.

concentrations were not amplified efficiently in the second PCR, as tube-to-tube variability was observed. Conversely, when the first PCR had more than 15 cycles we observed saturation when high template concentrations were used.

We next investigated whether preamplification was biasing quantitative estimates in cDNA extracted from normal cells. We first analyzed bias on highly expressed genes by studying mouse 28S (*Mrp-S21*) mRNA. We used serial dilutions of cDNA that was amplified in a double-round PCR (the first PCR was 15 cycles). We show that the correlation coefficient was high even when this highly expressed gene was tested, and high amounts of total cDNA were used (Fig. 4C). These results demonstrate that our double-round PCR conditions maintained linearity even when high template amounts were amplified.

We next analyzed possible bias in the amplification of rare messages. We studied tube-to-tube variability in mouse *Hprt-1* expression, considered a low-expressed gene (Pannetier et al. 1993). We retrotranscribed and amplified (15 cycles) *Hprt-1* of individual cells. Quadruplicates of the first PCR round were amplified in a second PCR. As shown in Figure 4D, quadruplicate aliquots of this message gave the same results, showing no tube-to-tube variability. We studied 15 individual cells using these conditions (data not shown), and all had the same quadruplicate C_T values, which also excluded the possibility of template switching inducing randomness and consequent tube-to-tube variability.

We next investigated whether we could reproducibly amplify two gene copies, by amplifying genomic DNA from individual cells. For that purpose, single cells were sorted, lysed, and processed for DNA extraction. In these conditions, mRNA is degraded and only DNA can be amplified. We used primer combinations spanning a small intron. In these conditions, amplicons generated after DNA amplification were only slightly longer than those generated when cDNA was amplified, ensuring that DNA and cDNA amplification had similar efficiency. We show that even with a two-copy template in the first PCR round, all samples amplified in the second PCR had the same C_T (Fig. 4E), excluding quantitative randomness effects and demonstrating the high sensitivity of our amplification procedure.

Finally, we tested the relative contribution of nonspecific signaling to our PCR read-outs. Indeed, SYBR Green incorporation in primers dimers could induce some type of background amplification. To evaluate this possibility, we studied several expresser and nonexpresser single cells for a particular gene product. As expected, some SYBR Green accumulation was sometimes detected in negative cells, but exponential accumulation (as found in positive cells) was never observed (Fig. 5A). Because C_T values are evaluated in the linear phase of SYBR Green accumulation, C_T determination in negative samples was not significantly different from samples where template was not included (Fig. 5B). These results demonstrate that negative cells did not originate significant background in our analysis.

In summary, these results demonstrate that our method of amplification preserves the initial representation of rare genes, simultaneously excluding excessive amplification of highly abundant gene copies and therefore allowing precise quantification assessments of copy numbers ranging from 1.28×10^9 to 2 molecules.

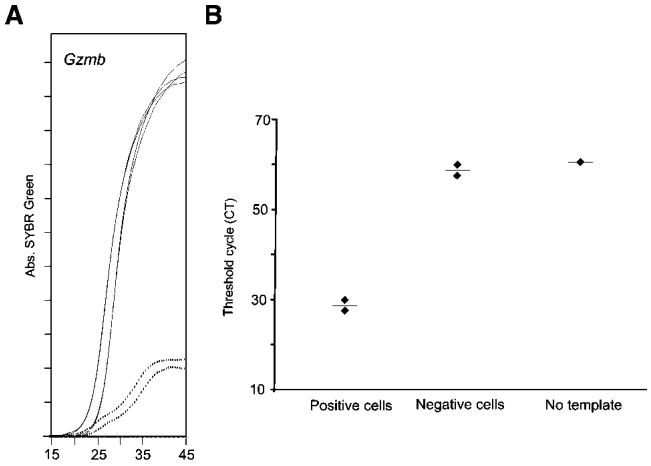

Figure 5 Impact of nonspecific signal in PCR quantification read-outs. Individual cells expressing or not *Gzmb* mRNA were amplified simultaneously. (*A*) SYBR Green signal in positive (solid lines) and negative (dotted lines) cells. (*B*) C_T evaluation, using the Sequence Detector 1.7 software. The C_T value of negative cells and wells not containing template was not significantly different (*t*-test: $P = 0.18$). This program's upper limit of detection is 60 cycles; that is, samples without template score with a C_T of 60.

Maintenance of Abundance Relationships

Exponential amplification has generally been considered to bias abundance relationships, as cDNAs of differing lengths and composition would be amplified with differing efficiencies (Freeman et al. 1999; Dixon et al. 2000; Phillips and Lipski 2000; Baugh et al. 2001). Our primers were designed to amplify cDNAs of similar length and composition, which should favor the maintenance of abundance relationships. To test directly how abundance relationships were maintained, different synthesized cDNA sequences (from mouse *Gzma*, *Gzmb*, and *Prf-1*) were quantified and mixed in known proportions (true ratios). These mixtures were then amplified in our two-step PCR. The C_T values obtained in the second PCR were used to determine the corresponding ratios after amplification (test ratios). To compare the maintenance of abundance relationships at very different template concentrations, these three templates were mixed at 1/1, 1/64, and 1/4096 ratios.

When the three sequences were all mixed at the 1/1 ratio, all PCRs had the same C_T (Fig. 6, upper left). When the different sequences were mixed at 1/1, 1/64, or 1/4096 ratios, differences of C_T values between different dilutions of different genes reflected initial dilutions, that is, six cycles for a 64-fold difference and 12 cycles for a 4096-fold difference. This occurred for all genes, tested in all types of ratio combinations (Fig. 6). Therefore, despite very different initial template proportions, our PCR procedure provided a measure (test ratios) that was faithful to the original template proportions. These data demonstrate that the maintenance of abundance relationships is guaranteed even on a large dilution range of the target template in a sample.

Reverse Transcription

To ensure maximal efficiency in capturing mRNA molecules present in individual cells, we used specific reverse transcription (RT), and the 5′ extreme of the amplified gene fragments of the first PCR was designed to be located between 300 and 400 bp from the 3′ RT origin (see Discussion). To validate our approach, we assessed the efficiency of the RT in these conditions. RNA fragments from different genes and respective complement cDNA sequences were produced and purified. We compared the direct amplification of a precise number of cDNA molecules with the amplification of the same number of synthesized RNA copies after their reverse transcription. We found that both cDNA and reverse-transcribed RNA template were amplified with similar efficiency (Fig. 7). This occurred when RNA templates coding for different genes were tested (Fig. 7A), and such efficient RT was detected at all RNA concentrations studied (Fig. 7B). These results demonstrate that we maintain copy numbers in the RNA to cDNA transition, and that this RT approach is highly efficient.

Population Versus Single-Cell Studies: The Impact of Single-Cell Analysis in the Evaluation of Functional Genetic Profiles

To compare the gene expression profiles obtained by single-cell analysis to those evaluated in bulk populations, we studied the same T-cell population using both methods. Mouse monoclonal CD8 T cells, 4 d after in vivo antigen stimulation (Tanchot et al. 1998; Veiga-Fernandes et al. 2000) were sorted either as 20 cells/well for population studies or as 20 single cells. All samples were retrotranscribed and amplified as described above. For simplicity, only four genes are shown.

Real-time PCR at a population level (Fig. 8A) showed a hierarchy of gene expression: *Gzmb* > *Tgf*-β = *Ifn*-γ > *Prf-1*. This data would suggest that *Gmzb* is the most expressed gene, and that this CD8 population differentiates similarly into *Tgf*-β- and *Ifn*-γ-expressing cells. In addition, these data suggest that these cells should be highly cytotoxic, due to the expression of high levels of *Gzmb* (an enzyme important for cytotoxicity) and express *Prf-1*. Indeed, CD8 killer activity requires the coexpression of both *Gzmb* and *Prf-1* (Russell and Ley 2002).

Results of single-cell studies revealed a very different scenario (Fig. 8B). First, most CD8 T cells differentiate into *Tgf*-β-expressing cells (17/20), whereas *Ifn*-γ and *Gzmb* expression was quite rare (4–6/20 cells). Moreover, *Gzmb* and *Prf-1* were usually expressed by different cells. These findings indicate that this CD8 population should be virtually devoid of killer activity, because individual cells do not coexpress the two genes required to kill target cells.

We conclude that single-cell multiparameter studies of gene expression reveal fundamental new insights into cell behavior. Conversely, the studies performed in bulk populations may be highly misleading.

DISCUSSION

The analysis of heterogeneity within cellular populations has a major impact on cell biology. The final aim of this approach is to reveal the gene expression patterns that ultimately characterize and define the fate of individual cells. Different cell fates likely rely on both qualitative and quantitative differences of gene expression that affect multiple genes simultaneously, but tests allowing the assessment of these features in individual cells are lacking. Here we describe a single-cell multiplex RT-PCR that allows simultaneous quantitative analysis and comparison of the expression of 20 genes in each individual cell. We demonstrate that this method substantially improves functional genomic read-outs. Conversely, quantitative studies performed at the population level may be very misleading.

It is not surprising that single-cell and population studies do not overlap. Quantitative studies at the population level only determine average rates of gene expression. They do not evaluate the frequency of expressing cells. The same mRNA amount can correspond to rare cells expressing high mRNA levels or to a much higher cell number expressing lower mRNA levels. These two situations may have very different biological meanings. The

impact of this potential bias has probably been underestimated, as the range of identical mRNA molecules each cell could express was not known. Here, for the first time, we were able to quantify messages at the single-cell level and found that expression of a single gene in individual cells could vary by 10,000 fold (data not shown). This extensive variation seriously undermines the interpretation of any quantitative studies that are not accompanied by frequency determinations. Indeed, in studies performed at the population level, very rare events (even at 10^{-4} frequencies) may score similarly to frequent events. Therefore, in population read-outs, events that are not representative of a global population behavior may appear as very significant events. This bias is evident in the study we include, where *Gzmb* expression appears to be a dominant function in the studied population, whereas studies at the single-cell level reveal that only a few cells expressed this mRNA.

Another major potential impact of single-cell studies is the possibility to determine gene coexpression. We were surprised to verify that different genes (*Prf-1* and *Gzmb*), which need to be coexpressed for CD8 cytotoxicity, could segregate into different individual cells. This finding emphasizes that studies at the population level are not sufficient to identify cell properties. Rather, coexpression studies at the single-cell level are fundamental for the interpretation of functional read-outs.

Concerning the present methodology, quantification of multiple gene expression in the same cell is only possible if several rules are followed simultaneously: the same efficiency of PCRs; the absence of primer and amplicon competition during the first PCR round, and an efficient RT to PCR transition requiring the use of specific RT. Moreover, all of these requirements are strictly interdependent.

Comparison of the expression of different genes between themselves requires that all individual PCRs have the same efficiency. This aim alone is not difficult to achieve. Primer combinations claiming to amplify multiple genes with similar efficiency are beginning to be available commercially. However, in these commercial kits (where controls for many important parameters are lacking), each individual gene must be studied separately, using one independent sample for each PCR. The ability to compare the expression of different genes between themselves and attribute coexpression of 20 genes to the same cell requires that all 20 different PCRs reactions be performed in the same tube and in the same PCR round. This imposes the requirement that, besides similar efficiency, the 40 primers and 20 amplicons of the first PCR also do not compete with one another.

It was claimed that analyses of more than five genes in one cell would necessarily lead to nonspecific inhibitions of amplification, which affect amplifications randomly (Walter et al. 2000). When using previous methods of single-cell amplification, we confirmed this claim, and all of our previous studies were restricted to four to five gene amplifications (Veiga-Fernandes et al. 2000; Lambolez et al. 2002). We found later that the modification of the PCR amplification conditions we describe herein, associated with a careful study of primer/amplicon competition, could prevent inhibition.

The constraints on primer selection impose another strategy: the use of specific reverse transcription which targets the mRNA sequence that is retrotranscribed and subsequently amplified. This is achieved by designing the 5′ extreme of the amplified gene fragments to be located between 300 and 400 bp from the 3′ RT origin. This strategy is both necessary and optimal for the maintenance of abundance relationships in the mRNA to cDNA transition.

The use of specific RT rather than poly-AAA reverse transcription is necessary to prevent 3′ bias that would modify abundance relationships in the transition from mRNA to cDNA. It is well known that poly-AAA reverse transcription preferentially transcribes mRNA fragments localized in the 3′ termini. This bias should be a major problem in our type of approach. Indeed, to ensure similar efficiency of amplification and prevent competition (essential aspects of our methodology), primers must be selected throughout the gene and not at the 3′ end only. Our strategy thus improves on previous methods used to achieve readings of gene expression in small samples such as modified poly-AAA reverse transcription methods (Dixon et al. 1998; Brail et al. 1999) or poly-AAA reverse transcription, followed by cDNA

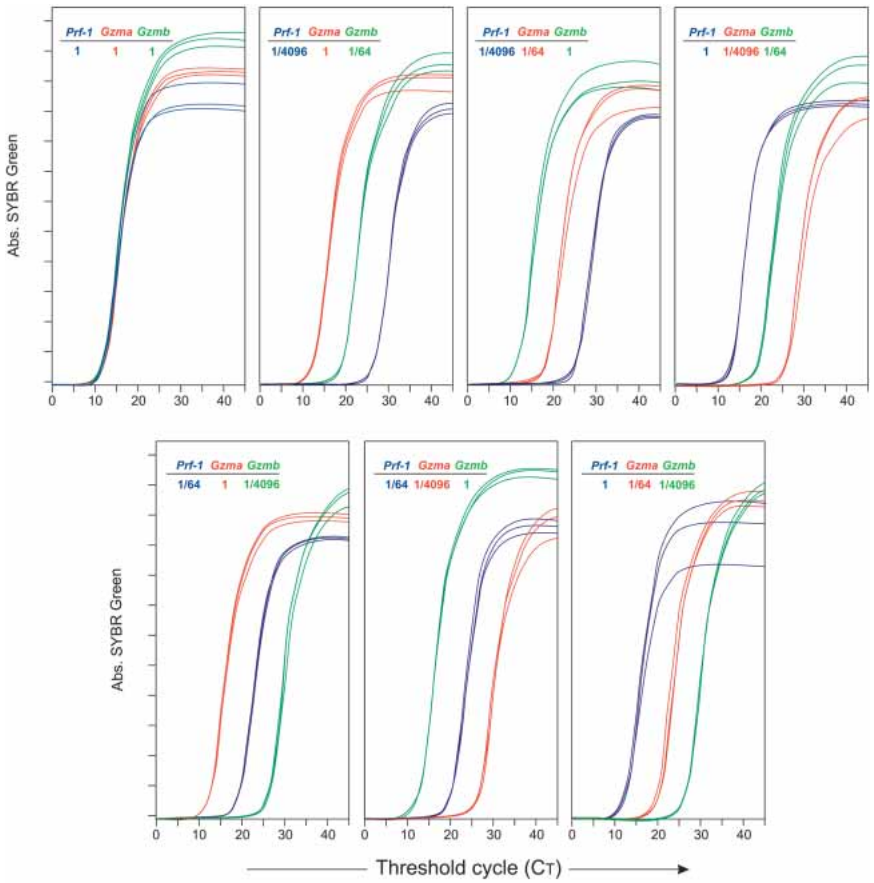

Figure 6 Maintenance of abundance relationships in double-step amplification. Different synthesized DNA sequences (*Prf-1*, blue; *Gzma*, red; *Gzmb*, green) were mixed in known proportions at different ratios indicated in each panel (true ratios). These mixtures were next amplified in quadruplicate by a two-step PCR of 15 preamplification cycles. Triplicates of amplification curves for each dilution are shown. C_T values between different dilutions of different genes reflected initial dilution conditions; that is, six cycles for a 64-fold difference and 12 cycles for a 4096-fold difference (data not shown).

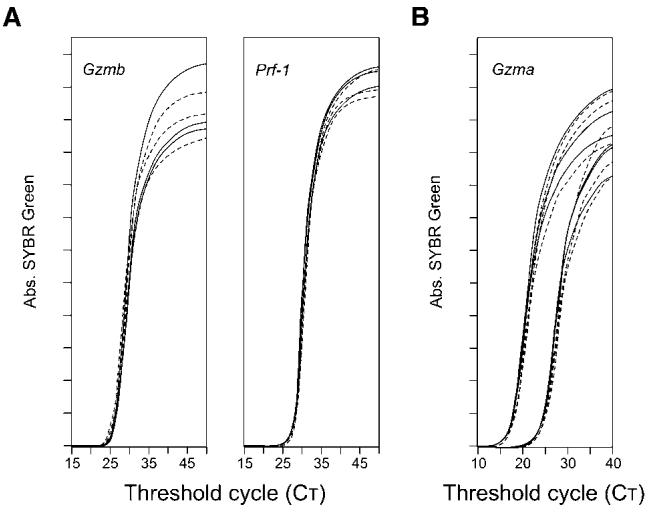

Figure 7 Efficiency of the reverse transcription. *Gzma*, *Gzmb*, and *Prf-1* RNA and DNA molecules were synthesized and purified. RNA was first retrotranscribed and subsequently amplified; DNA sequences were amplified directly following the same conditions as for cDNA. For each gene the same number of DNA and RNA molecules was compared. Results show triplicate amplifications of RNA (solid lines) and DNA (dashed lines). (*A*) Comparison of RT efficiency of the same number of RNA molecules coding for different genes. *Gzmb* (*left*) or *Prf-1* (*right*). (*B*) RT efficiency at different RNA concentrations. Different concentrations of *Gzma* RNA (ranging from 7.5×10^7 to 3×10^5 molecules) and corresponding DNA concentrations were compared. Results show RNA/ DNA amplifications at two of the concentrations tested: 1.9×10^7 and 4.7×10^6 molecules. The same results were obtained for all other concentrations.

polyadenylation at the 3′ and subsequent single-primer amplification of the tailed cDNAs (Brail et al. 1999). In contrast to our method, all of these other methods induce 3′-biased abundance relationships in the sample, which are difficult to control (Brail et al. 1999). Another limitation of poly-AAA RT methods is that the 5′ sequences of the retrotranscribed genes might be incomplete, compromising any further precise assessment. We prevented this limitation by designing the 5′ extreme of the amplified gene fragments to be located between 300 and 400 bp from the 3′ RT origin. We demonstrate that this strategy provides a maximized and uniform amplification of retrotranscribed genes, because RNA fragments are fully retrotranscribed and abundance relationships are maintained in the mRNA to cDNA transition. By comparing to a standard of RNA that is simultaneously reverse-transcribed and amplified, we can calculate the absolute number of mRNA molecules expressed per cell. These additional controls are lacking in all previous RT strategies that do not attempt to determine RT efficiency. A methodology was recently described that allows exponential amplification of cDNA yields, preserving the relative gene expression patterns of the initial sample (Iscove et al. 2002). However, this methodology also requires that gene-specific primers and probes are restricted to 3′ transcript termini (Iscove et al. 2002). Therefore, this strategy is also totally incompatible with multigene comparative quantification in individual cells that require primer and amplicon selection throughout the gene and not only on the 3′ end.

Because all efforts to achieve readings of gene expression in small samples were directed to increase cDNA yields, and thus are incompatible with gene coexpression studies, we used an alternative approach to measure the minute mRNAs recovered from one cell. Instead of generating very high amounts of cDNA, we exponentially amplified the low cDNA yields we obtained from each individual cell, and used this exponential amplification to quantify transcripts. It is usually assumed that this approach can bias the information content of the sample, as theoretical mathematic analysis showed that hybridization kinetics during thermal cycling could cause both sequence- and copy number-dependent bias (Peccoud and Jacob 1996). We show here that if adequate primer combinations are used, and optimized PCR conditions are applied, double-strand exponential amplification yields reproducible results from 1.28×10^9 to 4 copies of mRNA, which should cover all ranges of gene expression at the single-cell level.

This technique brings new perspectives to the understanding of biological processes. Most differentiation events have been studied on the basis of a population phenotype which does not necessarily reflect heterogeneity among the population. Conversely, single-cell analysis will allow further dissecting of cell decisions that ultimately influence a population phenotype. This technical approach also has a broader interest for diagnosis of minute samples. Indeed, in several pathologies and infections, only very small tissue samples can be obtained for diagnosis or continuous follow-up of disease progression. This method overcomes all restrictions in sample size by allowing the quantitative assessment of multiple different parameters from just a few cells. Indeed, we are presently using this method to characterize HIV-specific CD8 T cells that were divided into eight subtypes by cell surface markers, each subtype representing less than 0.1% of Peripheral Blood Lymphocyte (PBL). This approach allows the determination of 20 cell functions simultaneously, even in such small sample sizes. Preliminary evidence suggests that we will be able to quantify the expression of up to 40 genes/cell.

In conclusion, we here describe a method of quantitative multiplex PCR that can be applied to an extended number of genes expressed in a single cell. We also show that the ability to quantify multiple gene usage by individual cells provides fundamental insights into cell physiology and functional genomics.

METHODS

FACS Sorting

Cells were sorted using a FACS Vantage equipped with an automatic cell deposition unit (Becton Dickinson). Cells were collected in individual PCR tubes containing 5 µL of PBS-DEPC 0.1%, and stored at −80°C.

Reverse Transcription

Cells were lysed by cooling at −80°C followed by heating to 65°C for 2 min. After cooling to 4°C, RNA was specifically retrotranscribed for 1 h at 37°C by adding 10 µL of a mix containing 0.13 µM specific 3′ primers (see Supplemental Material I and II), 50 mM KCl, and 10 mM Tris-HCl at pH 8.3 (Applied Biosystems), 3.3 mM $MgCL_2$ (Applied Biosystems), 1 mM dNTPs (Pharmacia Biotech), 39 units of RNAse block (Stratagene), and 11.5 units of MuLV Reverse Transcriptase (Applied Biosystems), in a 15-µL reaction. The reaction was stopped by 3-min incubation at 95°C.

Primer Design

Gene sequence data and exon/intron boundaries were obtained from the Ensembl Project database (http://www.ensembl.org). The primers we selected for these PCR reactions are listed in Supplemental materials I and II.

Our primers were manually designed in order to avoid genomic amplification, by choosing 3′ and 5′ primers that hybridize with different exons. To achieve similar amplification efficiencies, we designed primers of 20 bp size targeting nonrepetitive sequences, with similar melting temperatures (T_m) calculated according to the formula (T_m = 64.9°C + 41°C × (number of G's and C's in the primer − 16.4)/number of bp of the primer) and amplifying fragments of a similar size. The composition of amplified fragments (50.61% ± 5.01% of GC content) was similar,

which is required to obtain uniform amplification efficiency for all different mRNAs. To prevent nonspecific amplification, all individual primer sequences were used in a BLAST search (http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html) of the mouse genome in order to check potential nonspecific hybridization of primers against other genes besides the targeted gene of interest. No significant hybridization was found with other genes.

To prevent primer competition, we selected primers and potential amplicons that did not cross-hybridize. Primer compatibility and size of the amplified fragments were assessed using the freely available software Amplify 1.2 (Engels 1993; http://engels.genetics.wisc.edu/amplify). The formation of primer dimers was also excluded, because the energies of primer associations that could lead to primer dimerization were considerably weaker than the 3′ binding energies of primer to template associations. Furthermore, the use of high annealing temperatures in our PCR protocol also contributed to exclude nonspecific amplification or inhibition. These aspects are of major importance, because during the first PCR round all genes are amplified simultaneously, and the disregard of such rules results in PCR inhibition.

First PCR Amplification

The cDNAs resulting from the reverse transcription reaction were next amplified. The first round of PCR consisted of one step of denaturation at 95°C for 10 min and 15 cycles of amplification (45 sec at 95°C, 1 min at 60°C, and 1 min 30 sec at 72°C) with 50 mM KCl, and 10 mM Tris-HCl at pH 8.3 (Applied Biosystems), 2 mM $MgCl_2$ (Applied Biosystems), 0.2 mM GeneAmp dNTPs (Applied Biosystems), 3 units of AmpliTaq Gold DNA Polymerase (Applied Biosystems), and 0.015 µM of specific primers (see Supplemental materials I and II) in an 85-µL reaction volume. When PCR was performed from single-cell genomic DNA, cells were previously treated for 45 min at 55°C and 10 min at 95°C with 7.5 µg of proteinase K (Merck) and 50 mM KCl, and 10 mM Tris-HCl at pH 8.3 (Applied Biosystems) in a final volume of 15 µL.

Real-Time Quantitative PCR

Real-time quantitative PCR was performed by adding 10 µL of 2× SYBR Green PCR Master Mix (Applied Biosystems) to each well containing 4 µL of template and 6 µL of a primer mix with 0.25 µM of each specific primer (see Supplemental materials I and II) in a 20-µL reaction volume using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). After a denaturation step at 95°C for 10 min, the cycle profile used was 30 sec at 95°C, 30 sec at 60°C, and 45 sec at 72°C for 60 cycles of amplification. An aliquot of 4 µL from the first PCR of positive cells was used to quantify the expression level of each different gene. Threshold cycle (C_T) was determined on the linear phase of PCRs using the software Sequence Detector version 1.7 (Applied Biosystems). PCR products were resolved on a 1.5% agarose ethidium bromide gel, and were all sequenced to confirm specificity (ABI PRISM 3100, Applied Biosystems). The PCR efficiency of each individual sample was assessed in the linear phase of a real-time PCR reaction using LinRegPCR version 7.0 software. This program uses the raw real-time PCR data of each individual sample and performs an assumption-free analysis (Ramakers et al. 2003).

Synthesis of Double-Strand DNA Sequences

cDNAs from *Prf-1*, *Gzma*, and *Gzmb* mouse genes were amplified using 5′-TCACACTGCCAGCGTAATGT-3′ and 5′-CTGTGGTAAGCATGCTCTGT-3′, 5′-TCAAATACCATCTGTGCTGG-3′ and 5′-AGAGGGAGCTGACTTATTGC-3′, and 5′-GTCAATGTGAAGCCAGGAGA-3′ and 5′-AGGATCCGATGTTGCTTCTG-3′, respectively. Amplified fragments were resolved on a 1.5% agarose ethidium bromide gel and purified using Wizard SV Gel and PCR clean-up System (Promega). DNA was quantified by incorporation of Picogreen (Molecular Probes) according to the manufacturer's instructions.

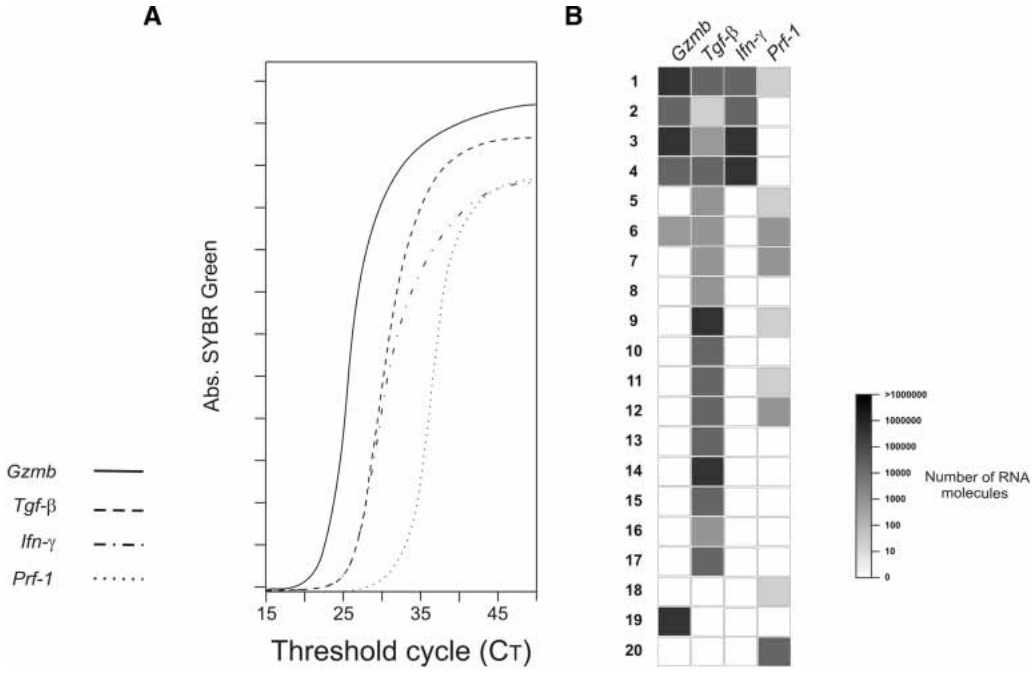

Figure 8 Impact of quantitative single-cell analysis on gene expression profiles. Monoclonal CD8 T cells, 4 d after in vivo antigen stimulation were sorted (*A*) at 20 cells/well for population studies, and (*B*) as 20 single cells at one cell/well. RT and PCR conditions were the same in both *A*,*B*. Results show: (*A*) Real-time PCR amplification of *Gzmb* (solid line), *Tgf*-β (dashed line), *Inf*-γ (dash-dotted line), *Prf-1* (dotted line). (*B*) Quantification of gene expression in individual cells, using quantitative single-cell multiplex PCR. Expression levels of each gene in each cell are shown as shades of gray, compared to the log scale in the *left*. The absolute number of mRNA molecules was obtained by comparing amplifications with a standard of a known number of RNA molecules that followed the same rules of RT and amplification.

Molecular Cloning and In Vitro Transcription

cDNA was obtained from RNA extracted from gut intraepithelial T lymphocytes (IELs) of C57Bl/6 mice and human small intestine using the RNeasy mini-kit (QIAGEN). We used these cells because they express all of the genes we studied. The cDNA was synthesized by incubating for 1 h at 37° C using 2.2 mM poly-(T) (Applied Biosytems) in a 45-µL volume reaction containing 50 mM KCl, 10 mM Tris-HCl at pH 8.3 (Applied Biosystems), 3.3 mM $MgCl_2$ (Applied Biosystems), 2.5 mM dNTPs (Pharmacia Biotech), 39 units RNAse block (Stratagene), and 3 units MuLV Reverse Transcriptase (Applied Biosystems). The reaction was stopped by 10-min incubation at 95°C. cDNAs from *Prf-1*, *Gzma*, and *Gzmb* mouse genes were first amplified using 5′-TCACACTGCCAGCG TAATGT-3′ and 5′-CTGTGGTAAGCATGCTCTGT-3′, 5′-TCAAATACCATCTGTGCTGG-3′ and 5′-AGAGGGAGCTGACT TATTGC-3′, and 5′-GTCAATGTGAAGCCAGGAGA-3′ and 5′-AGGATCCGATGTTGCTTCTG-3′, respectively. Amplified fragments were resolved on a 1.5% agarose ethidium bromide gel and purified using the Wizard SV Gel and PCR clean-up system. Sequences were next cloned as described (Poirel et al. 1997). Cloned fragments of *Prf-1*, *Gzma*, and *Gzmb* were used as templates for the in vitro transcription reaction. This reaction was performed using the MEGAscript T7 transcription kit (Ambion) using 1.5 µg of previously Hind III-linearized plasmid in a reaction volume of 80 µL. After transcription, the samples were treated with 4 units of DNase I for 15 min at 37°C and purified using the MEGAclear purification kit (Ambion). The purified RNA was eluted in 50 µL of Tris-EDTA, and an aliquot was run on a native gel (TBE1×, 2% agarose) and controlled for the presence of contaminating plasmid DNA and unfinished products of the in vitro transcription reaction using an Agilent 2100 bioanalyzer (Agilent Technologies) according to the manufacturer's instructions. In vitro transcriptions were >70% pure, and no DNA contaminations were detected. RNA and DNA were quantified by incorporation of Ribogreen and Picogreen (Molecular Probes) respectively, according to the manufacturer's instructions and using the ABI PRISM 7700 Sequence Detection System.

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MANUSCRIPT #2

Cartography of gene expression in CD8 single cells: novel CCR7^{-} subsets suggest differentiation independent of CD45RA expression

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Understanding the distribution, function and lineage relationship of CD8^{+} T-cell subpopulations is of fundamental value for the monitoring of the immune system in several experimental and clinical situations. However, the available data concerning the description of effector and memory CD8^{+} subsets in humans remains rather fragmentary since different studies favored the usage of distinct and restricted sets cell surface markers and functional parameters. We associated multiple markers to subdivide CD8^{+} T cells into fourteen different cell types several of which were not described previously, and evaluated the co-expression of eighteen genes simultaneously in individual cells from each subset. Our results show that each subset has a defined pattern of gene expression. Moreover, effector gene expression of CCR7^{-} cells correlated only to CD27 expression levels and CD27/CD28 co-expression, but not with CD45RA/R0 phenotypes. Our findings thus describe new CD8^{+} cell subsets, allow the identification of relatively homogeneous CD8^{+} subpopulations, provide a predictable and precise correlation between particular cell-surface markers and CD8^{+} T-cell functional properties and identify effector cells present in both the CCR7^{-}CD45RA^{+} and CCR7^{-}CD45R0^{+} compartments. The results also indicate that activated cells might modulate the expression of CD45RA/R0 asynchronously, rather than CCR7^{-}CD45RA^{+} cells always issuing from CD45RA^{-} precursors.

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The authors declare not having any financial interest related to the content of the present work. One of the authors (B.R.) holds a patent related to the work described in the present study. The single-cell multiplex RT-PCR method is covered by a patent deposed by the Necker Institute (patent number 0208593).

Abstract

Understanding the distribution, function and lineage relationship of $CD8^+$ T-cell subpopulations is of fundamental value for the monitoring of the immune system in several experimental and clinical situations. However, the available data concerning the description of effector and memory $CD8^+$ subsets in humans remains rather fragmentary since different studies favored the usage of distinct and restricted sets cell surface markers and functional parameters. We associated multiple markers to subdivide $CD8^+$ T cells into fourteen different cell types several of which were not described previously, and evaluated the co-expression of eighteen genes simultaneously in individual cells from each subset. Our results show that each subset has a defined pattern of gene expression. Moreover, effector gene expression of $CCR7^-$ cells correlated only to CD27 expression levels and CD27/CD28 co-expression, but not with CD45RA/R0 phenotypes. Our findings thus describe new $CD8^+$ cell subsets, allow the identification of relatively homogeneous $CD8^+$ subpopulations, provide a predictable and precise correlation between particular cell-surface markers and $CD8^+$ T-cell functional properties and identify effector cells present in both the $CCR7^-CD45RA^+$ and $CCR7^-CD45R0^+$ compartments. The results also indicate that activated cells might modulate the expression of CD45RA/R0 asynchronously, rather than $CCR7^-CD45RA^+$ cells always issuing from $CD45RA^-$ precursors.

Introduction

$CD8^+$ T lymphocytes play a key role in defense against cytosolic pathogens and tumors. Understanding the mechanisms through which the immune system controls such pathological situations to avoid disease depends upon the thorough characterization of all $CD8^+$ T subpopulations and differentiation stages, from naïve precursors to fully mature effectors. For that purpose, the $CD8^+$ T-cell compartment was subdivided into several different subsets with distinct properties. In humans, it has been established that expression of the lymph node homing receptor CCR7 can be used to separate both $CD4^+$ and $CD8^+$ $CD45RA^-$ T cells into two functionally distinct subsets: $CCR7^+CD45RA^-$ and $CCR7^-$ $CD45RA^-$, named "central memory" (T_{CM}) and "effector memory" (T_{EM}), respectively. Unlike $CD4^+$ T cells, an additional $CCR7^-$ subset that expresses CD45RA (T_{EMRA}) can also be found in the $CD8^+$ compartment. Since this population harbors cells expressing high perforin levels, it was suggested that the T_{EMRA} subset should correspond to a population of terminally differentiated $CD27^-$ effector cells, previously described by Hamann and collaborators [1]. These cells display a V_β repertoire significantly different from naïve cells, containing oligoclonal expansions of particular TCR V_β elements, and also have shorter telomeres, suggesting that $CD45RA^+CD27^-$ cells have been selected *in vivo* through antigen stimulation and evolved through extensive rounds of division [2]. The same authors had proposed an alternative classification for $CD8^+$ T lymphocytes in which CD45RA and CD27 expression was used to identify naïve ($CD45RA^+CD27^+$), memory ($CD45RA^-$ $CD27^+$) and effector ($CD45RA^+CD27^-$) $CD8^+$ T cells in humans [1]. This classification, however, underestimates the complexity of the memory $CD8^+$ T-cell subset revealed by the expression of CCR7. For instance, memory cells defined by the $CD45RA^-CD27^+$ phenotype would include both T_{CM} and T_{EM}, which were shown to enclose distinct functional specializations [3,4]. Furthermore, viral-specific $CD27^+CD8^+$ cells may also express CD45RA [5]. Heterogeneity of the effector/memory compartments was shown to be further extended to CD28 differential expression. $CD8^+$ T cells specific for several persistent human viruses were extensively characterized regarding their surface phenotype, perforin and granzyme A expression and *ex vivo* cytotoxic capacity. Based on these data, it has been shown that co-expression of CD27 and CD28 could be used to distinguish three functionally different subsets of $CD8^+$ T cells according to the progressive expression of effector functions: early ($CD27^+CD28^+$), intermediate ($CD27^+CD28^-$) and late ($CD27^-$ $CD28^-$) differentiated cells [5]. This classification thus reflects the activation status of antigen-experienced $CD8^+$ T cells, rather than discriminating effector and memory cells.

Moreover, CD27 and CD28 expression does not allow distinguishing T_{CM} from T_{EM}, nor T_{EM} from T_{EMRA}.

The prevailing data concerning the description of naïve, effector and memory $CD8^+$ T-cell populations in humans remains, thus, rather fragmentary. Manifestly, analysis of $CD8^+$ T cells including solely two or three parameters are not sufficient to reveal the whole heterogeneity of the antigen-experienced $CD8^+$ lymphoid compartment. The compound subsets are not clearly established, especially within the T_{EM} and T_{EMRA} compartments, and the correspondent differential roles and lineage relationships remain undisclosed. Interestingly, in different human chronic viral infections, as EBV, CMV, hepatitis C virus (HCV) and HIV-1, viral specific $CD8^+$ T cells display distinct predominant phenotypes [5-9]. However, it is not clear whether the lack of certain $CD8^+$ T subpopulations results from a specific virus-induced blockage in differentiation or, instead, is a consequence of different clinical settings[4,10]. Thus, understanding the distribution, function and relationship of the $CD8^+$ T-cell subpopulations is of fundamental value for the monitoring of the immune system in several experimental and clinical situations.

The present study aims to describe thoroughly the heterogeneity of the human $CD8^+$ T-cell compartment by establishing an accurate correlation between cell-surface phenotype and functional properties of each subset. In particular, this study characterizes the circulating human $CD8^+$ T cell populations based on the simultaneous association of CCR7, CD45RA, CD27, CD28, CD11a and CD62L cell-surface markers. The association of these particular markers allowed the identification of 14 different $CD8^+$ T-cell subsets, some of which never previously described. We isolated individual cells of each subset and studied in each cell the expression of 18 different mRNAs coding for cytokines, chemokines, cytotoxic molecules and several receptors. This work describes new $CD8^+$ T-cell subsets, identifies homogeneous $CD8^+$ T cell sub-populations and allows a predictable correlation between cell-surface phenotype and *in vivo* function. Moreover, it reports the presence of effector cells both in T_{EM} and T_{EMRA} subsets and suggests an asynchronous modulation of CD45RA/CD45R0 expression after priming, rather than $CCR7^-CD45RA^+$ cells always issuing from $CD45RA^-$ precursors.

Methods

Isolation of peripheral blood cells

PBMCs were obtained from healthy volunteers of both sexes with ages ranging from 22 to 56 years old after informed consent. Heparinized venous blood was centrifuged on J Prep density gradient (Adgenix) and $CD8^+$ T lymphocytes were purified from freshly isolated PBMC using the Dynal® CD8 Negative Isolation Kit (Dynal), according to the manufacturer's instructions. CCR7 depletion was performed by negative selection of cells labeled with purified anti-CCR7 (R&D Systems) using magnetic anti-IgG Dynabeads® (Dynal). Resulting $CD8^+$ $CCR7^-$ T cells were >98% pure.

Antibodies and reagents

Purified and FITC-labeled anti-human CCR7 were purchased from R&D Systems. PE-labeled anti-CD27 and APC-Cy7-labeled anti-CD8 were purchased from Ebiosciences. FITC-labeled anti-CD3 was purchased from Caltag. Streptavidin and other antibodies (CD62L anti-CD28, CD45RA, anti-CD4) were obtained from Pharmingen. They were directly coupled to FITC, allophycocyanin (APC), PE, PE-cyanine 7 (Cy7), APC-Cy7 or biotinilated, revealed by PE-Cy7- Streptavidin or APC-Cy7- streptavidin.

Cell sorting and flow cytometry

Cells were sorted using a FACS Vantage upgraded to DiVa configuration and equipped with an automatic cell deposition unit (Becton Dickinson). For single-cell sortings, cells were collected directly in 0,2 mL PCR tubes containing 5 µL of PBS 0,1% diethylpyrocarbonate (DEPC) and stored at -80ºC. Cells were analyzed in a BD-LSR I flow cytometer with CellQuest software (Becton Dickinson).

Primers and quantitative multiplex RT-PCR

Primer design was performed as described previously. Efficiency of amplifications for each gene and for each set of primers was calculated and proven to be maximal and uniform for all the genes in both first and second round of amplification. Competition was assessed and no interference was detected between the different primers and/or amplicons during multiplex amplification [11]. We used the following primers: RANTES/CCL5: 5'-CCTGCTGCTTTGCCTACATT-3', (sm)-5'-GTGCCCACATCAAGGAGTAT-3', rev-5'-TCCCAAAGTGCTGGGATTAC-3'; MIP-1α/CCL3: 5'-ACCTGCTCAGAATCATGCAG-3' (sm)-5'-CTCTCTGCAACCAGTTCTCT-3' rev-5'-TTCTGGACCCACTCCTCACT-3'; MIP-1β/CCL4: 5'-CTCACCTCTGAGAAAACCTC-3', (sm)-5'-TACCATGAAGCTCTGCGTGA-3', rev-5'-GATCAGCACAGACTTGCTTG-3'; granzyme A: 5'-CTCCTCATTCAAGACCCTAC-3', (sm)-5'-CTGCAGCTCACTGTAACTTG-3', rev-5'-CACATGGTTCCTGGTTTCACA-3'; granzyme B: 5'-CTTCCTGATACGAGACGACT-3', (sm)-5'-CCAGCAGTTTATCCCTGTGA-3', rev-5'-CTTGTTGCTAGGTAGCCTGA-3'; perforin: 5'-CCCTCTGTGAAAATGCCCTA-3', (sm)-5'-ACCAGCAATGTGCATGTGTC-3', rev-5'-GGAGTGTGTACCACATGGAA-3'; CD3: 5'-GGTTATTATGTCTGCTACCC-3', (sm)-5'-TGGAGATGGATGTGATGTCG-3', rev-5'-GGTCAGATGCGTCTCTGATT-3'; IL-2: 5'-CTCACCAGGATGCTCACATT-3', (sm)-5'-AACCTCTGGAGGAAGTGCTA-3', rev-5'-ACAATGGTTGCTGTCTCATC-3'; IL-10Rα: 5'-CCTAGAGATCCACAATGGCT-3', (sm)-5'-CGGGAAGATTCAGCTACCCA-3', rev-5'-TGCACTCCTCTTTAGACCAC-3'; IL-10:

5’-TGAAGGATCAGCTGGACAAC-3’, (sm)-5’-AGCCTTGTCTGAGATGATCC-3’, rev-5’-CACGGCCTTGCTCTTGTTT-3’; TGF-βR2: 5’-ACACTAGAGACAGTTTGCCA-3’, (sm)-5’-TGGAAGATGCTGCTTCTCCA-3’, rev-5’-GCTGATGCCTGTCACTTGAAA-3’; TGF-βR1: 5’-CCGTGAGGCAGAGATTTATC-3’, (sm)-5’-CAATGGTACTTGGACTCAGC-3’, rev-5’-TGCCAGTCCTAAGTCTGCAA-3’; IFN-γR2: 5’-CGACAGTAAATGGTTCACGG-3’, (sm)-5’-CACAGATCACAGCAACAGAG-3’, rev-5’-GGTATCAGCGATGTCAAAGG-3’; TNF: 5’-CTCTTCTCCTTCCTGATCGT-3’, (sm)-5’-CTCTCTCTAATCAGCCCTCT-3’, rev-5’-CTGGGAGTAGATGAGGTACA-3’; LTA/TNF-β: 5’-ACACCACCTGAACGTCTCTT-3’, (sm)-5’-TGTTGGCCTCACACCTTCA-3’, rev-5’-GGAAGGCACGGTCCGTGTTT-3’; IFN-γ: 5’-CTGTTACTGCCAGGACCCAT-3’, (sm)-5’-GGTCATTCAGATGTAGCGGA-3’, rev-5’-TGGATGCTCTGGTCATCTTT-3’; TGF-β1: 5’-GACAAGTTCAAGCAGAGTACA-3’, (sm)-5’-ACACATCAGAGCTCCGAGAA-3’, rev-5’-CACAACTCCGGTGACATCAAA-3’; Fas-L: 5’-CTGGTTGCCTTGGTAGGATT-3’, (sm)-5’-CAGCTCTTCCACCTACAGAA-3’, rev-5’-GGCAGGTTGTTGCAAGATTG-3’, (sm, semi-nested). Procedures for specific RT and multiplex semi-nested PCR are described in detail by [11]. PCR products were resolved on a 1.5% agarose ethidium bromide gel. An aliquot of 4 μL from the first PCR of positive cells was used to quantify the expression level of each different gene. Real-time quantitative PCR was performed by adding 12 μL of SYBR Green PCR Master Mix (Applied Biosystems) to each well containing 4 μL of template and 8 μL of a primer mix with 0,25 μM of each specific primer in a 24-μL reaction volume using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). After a denaturation step at 95°C for 10 min, the cycle profile used was 30 sec at 95°C, 30 sec at 60°C, and 45 sec at 72°C for 60 cycles of amplification. Threshold cycle was determined on the linear phase of PCRs using the software Sequence Detector version 1.7 (Applied Biosystems). Absolute quantification was extrapolated from a standard curve obtained following the amplification of triplicate serial dilutions containing known amounts of cDNA molecules and run in parallel with test samples in first and second PCR rounds.

Results

Cell-surface phenotypic characterization of the CD8$^+$ T-cell subsets from the human peripheral blood

We subdivided CD8$^+$ T-cell populations into four major subpopulations based on the expression of CCR7 and CD45RA, as previously reported[3,12] (Figure 1A). The co-expression of CD27, CD28 and CD11a (the alpha chain of LAF-1) was further evaluated within each cell subset. Both T_N (CD45RA$^+$CCR7$^+$) and T_{CM} (CD45RA$^-$CCR7$^+$) were quite homogeneous. Most co-expressed all these additional markers (Figure 1B, Table1). Furthermore, the intensity of expression of CD27, CD28 and CD11a did not vary within each cell subset (Table 2). In contrast, CCR7$^-$ subpopulations were very heterogeneous. Both T_{EM} (CD45RA$^-$CCR7$^-$) and T_{EMRA} (CD45RA$^+$CCR7$^-$) compartments contained T cells co-expressing CD28 and CD27 (double-positive, DP); expressing either one of these markers (single-positive, SP); or expressing none (double-negative, DN). Although the percentages of each T-cell subset varied between donors (Table 1), all these populations could always be found and presented the same characteristics in each individual donor (see bellow).

In some donors, we also found minor CCR7$^-$CD8$^+$ T-cell subsets expressing high levels of CD27 (CD27^{high}) (Figure 1B, Table1). These subsets were present in both T_{EM} and T_{EMRA} sets and were mainly composed by CD27-SP cells, but could also harbor DP cells (Figure 1B). In striking contrast to the other CD8$^+$ T cell subpopulations, the presence of CD27^{high} cells in blood appeared to be rather transitory, as the representation of these subsets in the same donor varied significantly with time.

It was previously reported that *in vitro* activation of CD8$^+$ T cells induces down-regulation of CD27 and CCR7 [3,13,14] and up-regulation of CD28 and CD11a [15,16]. Accordingly, T_N cells had the highest levels of CD27 and CCR7 (Figure 1A, 2A), as compared to T_{CM}, while T_{EM}- and T_{EMRA}-27SP expressed even lower levels of CD27 than T_{CM}. CD28 and CD11a followed the opposite trend: CD28 expression was lower in T_N than in all subsets of primed cells and CD11a was upregulated from T_N to $T_{CM} < T_{EM} < T_{EMRA}$ (Figure 2B,C and Table 2). However, within the T_{EM} and T_{EMRA} subsets, cells expressing CD27 and/or CD28 (DP or SP) displayed similar expression levels of these markers, as well as identical high levels of CD11a (Figure 2). Interestingly, independently of their additional phenotype, CD27^{high} cells expressed relative low levels of CD11a when compared to any other subset of primed cells (Figure 2C and Table 2). It was previously reported that CD27 could be

transiently up-regulated shortly after *in vitro* activation. The relatively low CD11a expression of $CD27^{high}$ cells and their transitory presence in the blood suggest they may be recently activated $CD8^+$ T cells. Therefore, CD27, CD28 and CD11a expression levels confirm the putative activation hierarchy $T_N < T_{CM} < CCR7^-CD8^+$ T cells, but do not allow further discrimination within the complex $CCR7^-$ compartment. $CD27^{high}$ cells behave as a distinctive sub-population, with CD11a levels approaching those of naïve cells.

Expression of CD62L within the $CCR7^-$ compartment is highly restricted to $CD27^{high}$, DP and CD28-SP subsets

CD62L plays a fundamental role on the migration of lymphocytes to secondary lymphoid organs. While T_N and T_{CM} $CD8^+$ T cells are consistently $CD62L^+$, only a fraction of $CCR7^-$ cells expresses this adhesion molecule [3].We have further investigated if CD62L expression was related to peculiar $CCR7^-CD8^+$ T-cell subtypes (Figure 2D). We found a correlation to CD27/CD28 expression, but no differences between T_{EM}/T_{EMRA} subsets. Thus, independently of their CD45RA phenotype, either $CD27^{high}$, DP or CD28SP cells contained abundant $CD62L^+$ cells. Only a minor fraction of CD27SP cells expressed low levels CD62L. CD62L expression in DN cells was even lower. It is unlikely that such low expression might be sufficient to ensure migration to the lymph nodes, since we could never detect DN $CD8^+$ T populations in lymph node cells (unpublished data).
Altogether, these observations revealed that T_N and T_{CM} are homogeneous populations with respect to all additional markers, whereas T_{EM} and T_{EMRA} are significantly heterogeneous, containing multiple subpopulations that likely cross a large spectrum of activation status.

$CD8^+$ T-cell subpopulations heterogeneity evaluated at a single-cell level

The further addition of CD27 and CD28 to previous established CD45RA and CCR7 cell surface markers subdivided the $CD8^+$ T-cell compartment into fourteen phenotypically different cell subsets (Figure 1). Whether this subdivision is sufficient to fully describe the functional properties of $CD8^+$ T cells, or each of the individual $CD8^+$ T-cell subsets can be yet heterogeneous, harboring cells with multiple functional potentialities is not known. To evaluate the homogeneity of $CD8^+$ T-cell populations, we envisaged to isolate individual cells from each $CD8^+$ T-cell subset by cell sorting and analyze multiple gene expression in each individual cell by a method of multiplex RT-PCR we developed. This method allows

to assess in each individual cell the simultaneous expression of genes coding for inflammatory chemokines, cytokines, cytotoxic molecules and several receptors described to be involved in $CD8^+$ T-cell responses. To minimize the chances of include cellular contaminants in our study, we have purified $CD8^+$ T lymphocytes by magnetic negative selection prior to sorting, and we have further assessed in each sorted cell the presence of the mRNA coding for CD3. Thus, these data correspond exclusively to $CD8^+$ T cells expressing the CD3 mRNA.

We found that some genes (IL-2, IL-10 or MIP-1α/CCL3) were expressed in such low frequencies (less than 5%) that their impact on functional profiles could not be analyzed at single-cell-level. Also some of these sub-populations were so rare (less than 0.05% of CD8 cell sets) that could not be sorted reliably. For this reason, we failed to characterize $CD27^{high}$-DP cells, and could only collect CD28-SP from one single donor. For all remaining 11 populations we have characterized fifteen different parameters in each individual cell. To evaluate if cell-surface phenotypes always correlated to peculiar functional profiles, we compared the gene expression profiles of three different individuals. We found that within each phenotype gene expression profiles were remarkably similar between all donors. When some variation was found, the ranges of expression are mentioned in the text.

T_N cells lack effector functions, but express several receptors types

As expected, the less activated $CD8^+$ T-cell set was T_N (Figure 3A). These cells did not express mRNAs coding for chemokines, cytotoxic molecules or effector cytokines, such as TNF-α or IFN-γ. However, a small fraction of cells (<15%) generally expressed TNF-β (coded by the *lta* gene). In addition, about 40-50% of the cells expressed TGF-β1 and most expressed TGF-β receptor 2 (TGF-βR2). In addition to TGF-βR2, TGF-βR1 expression is also required for TGF-β1-induced signaling to occur. Co-expression of TGF-βR1 and TGF-βR2 was detected in more than 30% of the naïve cells. T_N population also contained the highest frequency of cells expressing IFN-γR2, which determines the responsiveness to exogenous IFN-γ, and approximately one half of the cells expressed interleukin IL-10Rα. Interestingly, these results are coincident with the expression patterns found in mouse naïve $CD8^+$ T cells, although TGF-β1 is then expressed in much lower frequencies (Peixoto et al., submitted for publication).

Cells expressing CD27^{high} display the gene expression pattern more closely related to T_N cells

Surprisingly, the CD8$^+$ T-cell subpopulations that most resembled T_N were the T_{EM}-CD27^{high} and T_{EMRA}-CD27^{high} subsets (Figure 3B). These populations expressed TGF-β1 at the same frequency as naïve cells and TNF-β was expressed in a slightly lesser extent. In contrast, IFN-γR2 was no longer expressed, a finding we observed in all antigen experienced CCR7$^-$CD8$^+$ T-cell subsets. IL-10Rα frequencies increased up to 75% and perforin and granzymes were expressed in average by 20% and 10% of the cells, respectively. However, expression of perforin and either granzyme A or B was detected in separate cells, suggesting that cells expressing CD27^{high} are not cytotoxic. Remarkably, the mRNA coding for the inflammatory chemokine RANTES (also known as CCL5) was detected in 45-70% of CD27^{high} cells. Importantly, T_{EM} and T_{EMRA} CD27^{high} subsets had undistinguishable gene expression profiles, suggesting they might play similar functional roles *in vivo*.

T_{CM}: the memory subset expressing fewer effector functions

T_{CM} cells, although expressing CCR7, displayed higher frequency of effector genes than CCR7$^-$CD27^{high} subpopulations (Figure 3C). Three evident differences were noticed. First, the mRNA coding for granzyme A (GZMA) was up-regulated, since it was expressed in up to 40% of the cells. However, only a very modest percentage of T_{CM} cells (<15%) co-expressed this molecule along with perforin, indicating that only a small fraction of these cells can be cytotoxic. Secondly, expression of RANTES was also up-regulated and could be detected in more than 70% of the cells. Finally, expression frequency of the IFN-γR2 was down-regulated being detected but in a small fraction of cells. It must be noted that only T_N and T_{CM} subsets do express IFN-γR2 mRNA, all other sets of activated T cells lacking this molecule.

T_{EM} and T_{EMRA} harbor three hierarchically differentiated subsets

The gene expression pattern of all CCR7$^-$ subpopulations, with the exception of the previously described CD27^{high} subsets, shows a degree of functional differentiation significantly higher than the T_{CM} subset (Figure 4). This was revealed by increased

expression of RANTES, which is consistently expressed my more than 90% of the cells, perforin, GZMA and IL-10Rα, together with further expression of additional molecules.

Within $CCR7^-$ cells, the DP cellular subsets were those more closely resembling the T_{CM} subset. As compared to T_{CM} cells, virtually all T_{EM} and T_{EMRA}-DP now expressed RANTES, GZMA and IL-10Rα, and perforin was expressed in much higher frequencies ranging from 50 to 80% (Figure 4A). In a fraction of these cells, we detected for the first time expression of Fas-L (6-20%) and macrophage inflammatory protein (MIP)-1β, also named CCL4 (14-40%). Only very rare cells could score positive for IFN-γ or granzyme B (GZMB). Strikingly, the patterns of gene expression of T_{EM} and T_{EMRA}-DP populations were nearly overlapping, the sole difference concerning a slight increase on the frequencies of perforin and FAS-L expression in T_{EM}-DPs. We were able to isolate CD28SP cells from a single donor and solely of the T_{EM} compartment. Interestingly, this subset displayed a pattern of gene expression very similar to the DP subpopulations (Figure 4B), suggesting a close relationship.

The T_{EM} and T_{EMRA} CD27-SP subsets were more activated than the DP subsets (Figure 4C). Indeed, in addition to the genes already expressed at high frequency by DP cells, GZMB expression was up-regulated (10-50%), while the frequency of Fas-L expression shows a less striking increase (15-25%). Once again, we were surprised to notice an evident overlap between the expression patterns of T_{EM} and T_{EMRA}-CD27-SP subsets that was extended to all the molecules we have studied.

T_{EM} and T_{EMRA}-DN $CD8^+$ T cells displayed the most activated gene expression profile (Figure 4D). Now, near all cells expressed perforin and GZMA, GZMB was expressed by most cells and Fas-L expression frequencies increased. In some donors, IFN-γ expression was also up-regulated, being detected in up to 30% of the cells. Again, we found no difference in gene expression profiles between T_{EM} and T_{EMRA}-DN $CD8^+$ T cell sets.

Thus, the results obtained by single-cell multiplex RT-PCR clearly depict a hierarchy of T-cell differentiation status in antigen-experienced cells. Importantly, this hierarchy is defined by CCR7 expression, CD27 expression levels and CD27/CD28 co-expression, but does not correlate with expression of CD45RA.

Gene expression of T_{EM} and T_{EMRA} compounding subsets is similar at quantitative level

Since the gene expression profiles of all $CCR7^-$ T-cell subsets correlated to CD27/CD28 expression but not to $CD45RA^+/CD45RA^-$ phenotype, we next investigated if we could distinguish T_{EM} and T_{EMRA} cell sets by a different amount of effector mRNA gene

expression. This approach was possible because in the methodology used here the efficiency of reverse transcription was evaluated [11]. Therefore, we were able to directly quantify the number of mRNA molecules coding for distinct genes in each individual cell. Moreover, our methodology also uses PCR reactions of identical efficiency for all genes, allowing the comparison of different relative gene expression levels. We have thus quantified the expression of all genes in all gene expressing cells from different populations in the same donor. As expected, not all the genes were expressed at the same level. Perforin and MIP-1β had the lowest number of mRNA molecules per cell, while RANTES and the receptors for TGF-β and IL-10R displayed the highest level of expression levels (not shown). Nevertheless, the expression level of each gene was relatively constant between the different cell subsets (Figure 5). Moreover, T_{EM} and T_{EMRA} showed overlapping gene expression levels in all individual cells we tested, as exemplified for two of the genes in Figure 5. These results further support that CD45RA expression can not discriminate $CCR7^{-}CD8^{+}$ T-cell subtypes neither at qualitative or quantitative levels.

Discussion

The human $CD8^+$ T-cell compartment encloses several subpopulations with multiple functionalities, including naïve, effector and memory subsets. The prevailing data describing these subsets in the peripheral blood is unclear in several aspects, mainly due to multiple analyses of $CD8^+$ T cell subpopulations using different and limited sets of surface markers and functional properties. The present study aims to elucidate the ambiguous and missing data concerning the heterogeneity of the human $CD8^+$ T-cell compartment. For this purpose, we used two approaches. Firstly, we performed a detailed characterization of the cell-surface phenotype of the circulating $CD8^+$ T cell populations *ex vivo*, based on the simultaneous association of the most common and relevant cell-surface markers described in the literature, namely CCR7, CD45RA, CD27, CD28, CD62L and CD11a. These molecules are widely used to identify $CD8^+$ T-cell subsets, but they are usually only partially associated [1,3-5,12]. The concurrent association of all these cell-surface markers allowed the identification of multiple $CD8^+$ T cell subsets, several of which were never described previously. Importantly, we could directly compare the primed subsets present in the $CD45RA^+$ and $CD45RA^-$ compartments, whose precise differential functions remained unclear thus far. The second strategy concerned an approach to evaluate the heterogeneity of each one of these cellular subsets. We studied individual cells in each population and each cell was characterized for the expression of 18 different mRNAs involved in T-cell functions. Single-cell gene expression analysis allowed the assessment of functional heterogeneity inside each cell subset and gave important insight concerning the differential function of the various subpopulations. Furthermore, we also found that each one of these particular phenotypes corresponded to specific patterns of gene expression, since each displayed reproducible gene expression patterns in all the donors studied. Thus, the phenotypes we here describe apparently can be used to predict defined characteristics in $CD8^+$ subpopulations in normal healthy individuals.

Our results show that the combination of CCR7, CD45RA, CD27 expression levels and CD28 permits to discriminate 14 $CD8^+$ T-cell subsets. With the exception of $CD27^{high}$ cells, which display characteristics of recently activated populations, all remaining subsets could be found in all donors, albeit with different representations. When isolated and studied at single cell level, each subset displayed a characteristic pattern of gene expression. In $CCR7^-$ cells, this pattern strongly correlated to expression of both CD27 and CD28, following a hierarchy of cell activation $CD27^{high}$ < DP < CD28-SP < CD27-SP < DN. Surprisingly within each of these subsets, $CD45RA^-$ T_{EM} cells showed the same gene

expression patterns, at both qualitative and quantitative level, as their counterparts expressing CD45RA. These findings contradict the paradigm that T-cell activation necessarily leads to CD45RA loss, and that further maturation induces CD45RA re-expression in such way that effector cells should be present only in the T_{EMRA} compartment. They rather suggest that $CD8^+$ T cells may modulate or not CD45R0/RA expression after activation, rather than T_{EMRA} cells forcedly differentiate from T_{EM} precursors.

Indeed, we clearly found fully differentiated cells co-expressing multiple "killer" genes in both $CD45RA^+$ and $CD45RA^-$ populations. Our results on the study of $CD27^{high}$ subpopulations also support that CD45RA isoform can be maintained after T cell activation. It has been reported CD27 is transiently up-regulated after T-cell activation *in vitro*, the peak expression level occurring by 24 hours [17,18]. Our results strongly support that CD27 is also up-regulated following *in vivo* activation, since $CD27^{high}$ cells have all the characteristics of recently activated cells: they were not detected in all donors and their frequency in the same donor was not stable, suggesting that they may disappear with time. Furthermore, the gene expression profile of these $CD27^{high}$ subpopulations was very close to that of naïve cells. As major difference, a fraction of $CD27^{high}$ cells expressed RANTES, which has been reported as being one of the first genes to be induced after *in vitro* activation. It was described to be already up-regulated 3 to 5 days after T cell activation [19-21]. However, "recently activated" $CD27^{high}$ cells with the same characteristics can be found in both T_{EM} and T_{EMRA} populations, indicating that primed populations can maintain CD45RA after activation. Other independent evidence suggests T_{EMRA} populations can derive directly from naïve cells, since their replicative history may approach that of naïve T cells [12]. It is possible that besides CD45R0/RA, activated T cells may alternatively maintain or loose CD28/CD27. We could detect recently activated $CD27^{high}$ cells that co-expressed both molecules, as well as primed cells that were CD28-SP cells.

Since our results argue against the model of a mandatory T_{EMRA} origin from T_{EM} cells, it is adequate to review the experimental evidences leading to this notion. The CD45R0 phenotype was believed to be characteristic of primed cells, but the detection of $CD8^+$ $CD45RA^+$ cells with all characteristics of effector cells forced to review this issue. *In vivo* analysis of antigen specific cells for persistent human viruses, commonly HCMV and EBV, showed that at early time points of acute infections epitope-specific $CD8^+$ T cells were prevalent in the $CD45R0^+$ subset, but in the chronic phase both $CD45RA^+$ and $CD45R0^+$ $CD8^+$ T-cell subsets contain significant frequencies of cells with the same specificity [22,23]. It was therefore assumed that after the primary response, some of the

clonally expanded CD45R0$^+$ viral-specific CTLs revert into a memory CD45RA$^+$ phenotype [23]. Nevertheless, this hypothesis was never fully confirmed. In the primary immune response to EBV infection, a frequency of 5-14% of EBV-specific cells yet expresses the CD45RA isoform early after infection and the CD45RA/R0 distribution of individual clones was not investigated [22]. Wills and collaborators analyzed the distribution of a single CMV-specific clone in only two donors and always found the same clone in both CD45RA/R0$^+$ subsets, albeit at different frequencies. However a more extensive study investigating CD45 isotype expression and the TCR V_β usage showed that the dominance of the CD45 phenotype was extremely variable between individuals, as in some cases the immunodominant clone was predominantly CD45RA$^+$ and in others CD45R0$^+$ [24]. Finally, the replicative history of the CD45RA$^+$ CD8$^+$ T-cell subpopulations supports the idea that those cells can differentiate directly from the naïve pool and, thus, a CD45R0$^+$ stage is not necessary [12]. Furthermore, recent *in vitro* studies failed to induce CD45RA re-expression in T_{EM} cells, while T_{CM} cells re-expressed CD45RA exclusively under cytokine influence, but never after T cell triggering. It is therefore clear that more thoroughly *ex vivo* studies are required to determine the lineage relationships between these cell types, possibly through the evaluation of the distribution of multiple clones in several independent donors.

The possibility that CCR7$^-$CD45RA$^+$ CD8$^+$ T lymphocytes can issue directly from naïve cells [12] is in apparent contradiction with other data suggesting that T_{EMRA} corresponds to a terminal differentiation stage, in contrast to T_{EM}, given the highest perforin content and reduced division capacity [3,4]. It must be noted that in these later studies CD45RA$^+$ and CD45RA$^-$ cells were not subdivided based on CD27 and CD28 expression. Our results clarify these apparent contradictory data. We show that T_{EM} and T_{EMRA} have different distribution of the compounding subsets DP, CD27-SP, CD-28SP and DN. DN cells, the most activated CD8$^+$ T-cell subset, are enriched in the CD45RA$^+$ compartment, explaining why this subset appeared to harbor more differentiated cells in previous studies. These results emphasize the importance of the additional characterization of CCR7$^-$ cells through assessment of CD27 and CD28 co-expression.

We also show that each subset within T_{EM} and T_{EMRA} CD8$^+$ T-cell subpopulations is characterized by the acquisition of a particular effector function. Interestingly, this phenomenon appears to occur sequentially, in such a way that along the hierarchy of activation, the expression of each gene once induced is never lost in the subsequent activation stages. Hence, the DP subsets had high frequencies of cells expressing RANTES and GZMA, and a low perforin expression, whereas CD27SP cells maintained high

frequencies of RANTES and GZMA, but now up-regulated perforin, rare cells expressing GZMB. Subsequently, DN cells co-expressed high frequencies of RANTES, GRANZA and perforin but now also up-regulated GZMB, and Fas-L expression. These results demonstrate that differential cellular activation leads to the progressive co-expression of multiple "killer" mRNAs by the same cell. Since each of these molecules mediates killing by a different mechanism, their co-expression may occur to improve killer efficiency of individual cells. Indeed, association of granzymes and perforin is fundamental for perforin-mediated cytotoxicity [25] and FasL cytolysis alone is not very efficient [26]. In humans, it was shown that different viral infections selectively induce a preferential differentiation of cells to distinct phenotypes [5]. EBV induces DP cells, HIV CD27-SPs and CMV generates DN types. These differential phenotypes were attributed to a different capacity of lymphocytes to become fully activated. Nevertheless, all these infections induce major expansions of $CD8^+$ T lymphocytes in the acute phase and infection by EBV is well controlled by DP cells. Thus, an alternative possibility is that the immune response to each virus requires the generation of particular effector subsets. Actually, since all T_{EM} and T_{EMRA} populations (with the exception of $CD27^{high}$ subsets) co-express perforin and granzymes and are able to mediate cytotoxicity, it is possible that the panel of molecules expressed by each one $CD8^+$ T-cell subpopulation confers particular advantages for the control of each type of infections. Therefore, the predominance of a given phenotype amongst viral-specific $CD8^+$ T cells can result from the selection of the most advantageous $CD8^+$ T-cell subset in the control of each type of infection.

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Table 1. CD27 and CD28 co-expression in $CD8^+$ $CCR7^-$ T cells

%	$CD27^+CD28^+$ DP	$CD27^+$ SP	$CD28^+$ SP	$CD27^-CD28^-$ DN	$CD27^{high}$ DP	$CD27^{high}$ SP
$CD45RA^-$	47 (30-65)	16 (2-27)	8 (2-15)	22 (7-46)	1,5 (0,6-2)	5,5 (1-13)
$CD45RA^+$	21 (10-44)	19 (7-28)	6 (2-12)	47 (19-72)	2 (0,9-3)	5 (2-15)

Results are from 8 healthy donors. They depict the average distribution of different $CCR7^-$ T_{EM} or T_{EMRA} subpopulations expressing CD27 and/or CD28 and, in brackets, the minimum and maximum values. DP, double-positive; SP, single-positive; DN, double-negative.

Table 2. Expression levels of different markers in $CD8^+$ T cells

	CD27	CD28	CD11a
N	445	30	164
T_{CM}	270	74	513
T_{EM}	153	66	608
T_{EMRA}	119	51	628
$CD27^{high}$	542	37	181

Results show mean fluorescence intensity (MFI) of stainings for different cell surface markers gated on positive populations in PBMCs of one donor. Similar relationships were found in other donors.

Figure Legends

Figure 1. CD27/CD28 expression in $CD8^+$ T peripheral blood lymphocytes. PBMCs were subdivided using CCR7 and CD45RA expression in naïve (T_N) central memory (T_{CM}) effector memory (T_{EM}) and effector memory $CD45RA^+$ (T_{EMRA}) cell subpopulations (A) and CD27/CD28 co-expression evaluated in each of these cell sets (B).

Figure 2. Expression levels of different markers in different $CD8^+$ T-cell subsets. *Upper panels:* comparison of CD27 (A) or CD28 (B) and CD11a (C) expression levels on T_N cells (shadowed histograms) with: T_{CM} (left); T_{EM}*:* (thick line) T_{EMRA} (dotted line). On (C) left graph includes CD11a expression on $CD27^{high}$ cells (dashed line). *Lower panels (D)*: T_{EM} and T_{EMRA} subpopulations were subdivided accordingly to their expression of CD27 and CD28 into $CD27^{high}$, $CD28^+CD27^-$ (CD28-SPs), $CD28^+CD27^+$ (DP) $CD28^-CD27^+$ (CD27-SP) and $CD28^-CD27^-$ (DN) cell sets. Results show CD62L expression in each of these sets in one representative donor.

Figure 3. Single-cell gene expression profiles of the less activated $CD8^+$ T-cell subpopulations. $CD8^+$ T cells (A) naïve, (B) $CCR7^-CD27^{high}$ $CD45RA^+$ and $CD45RA^-$ (C) T_{CM} were single-cell sorted and the expression multiple genes was determined simultaneously in each individual cell. Each horizontal line depicts the same cell individual cell that is numbered. Each vertical line shows a different gene. All cells tested were positive for the expression of CD3ε mRNA. Results depict representative profiles of three donors studied, with overlapping results. The following genes *ccl5*, *ccl4*, *tgfb1*, *tnf*, *lta*, *ifng*, *prf1*, *gzma*, *gzmb*, *faslg*, *tgfbr1*, *tgfbr2*, *ifngr2* and *il10ra* code, respectively, for RANTES, MIP-1β, TGF-β1, TNF-α, TNF-β, IFN-γ, perforin, granzyme A, granzyme B, FasL, TGF-β receptor 1, TGF-β receptor 2, IFN-γ receptor 2 and IL-10 receptor α.

Figure 4. Single-cell expression profiles of the most differentiated $CD8^+$ T-cell subpopulations. Single cells of each $CD8^+$ T-cells subset were sorted individually and a multiplex RT-PCR was performed as for Fig. 3. Each single cell corresponds to one line, whereas the expression of the several genes studied is depicted vertically. The representative gene expression patterns presented correspond to (A) the DP, (B) CD28-SP, (C) CD27-SP and (D) DN $CCR7^-CD8^+$ T subsets. Results depict representative profiles of three donors studied, with overlapping results, with the exception of CD28-SP for which

one single donor was studied. The following genes *ccl5*, *ccl4*, *tgfb1*, *tnf*, *lta*, *ifng*, *prf1*, *gzma*, *gzmb*, *faslg*, *tgfbr1*, *tgfbr2*, *ifngr2* and *il10ra* code, respectively, for RANTES, MIP-1β, TGF-β1, TNF-α, TNF-β, IFN-γ, perforin, granzyme A, granzyme B, FasL, TGF-β receptor 1, TGF-β receptor 2, IFN-γ receptor 2 and IL-10 receptor α.

Figure 5. Quantitative assessment of mRNA expression in single cells. Single cells of each $CD8^+$ T-cell subsets were sorted and cells expressing each gene identified, as shown in Fig.3, 4. In all the positive cells shown in these figures, the mRNA expression levels were further quantified. Each graph shows three representative cells from each population. We show GZMA and RANTES amplification since these genes can be found in all cell sets, and thus their expression levels can be directly compared. We found that different genes were expressed at different levels, but expression of each gene was similar in cells belonging to different CD8 sub-populations.

Figures

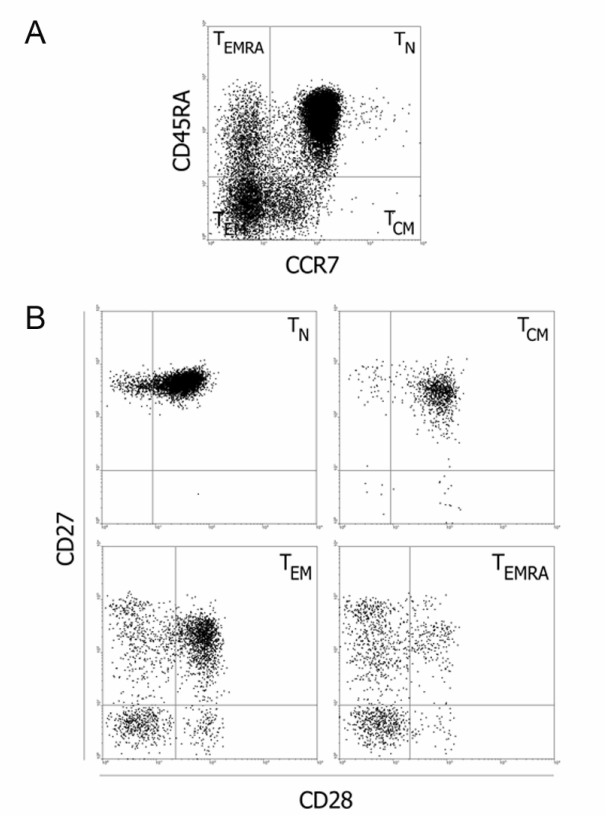
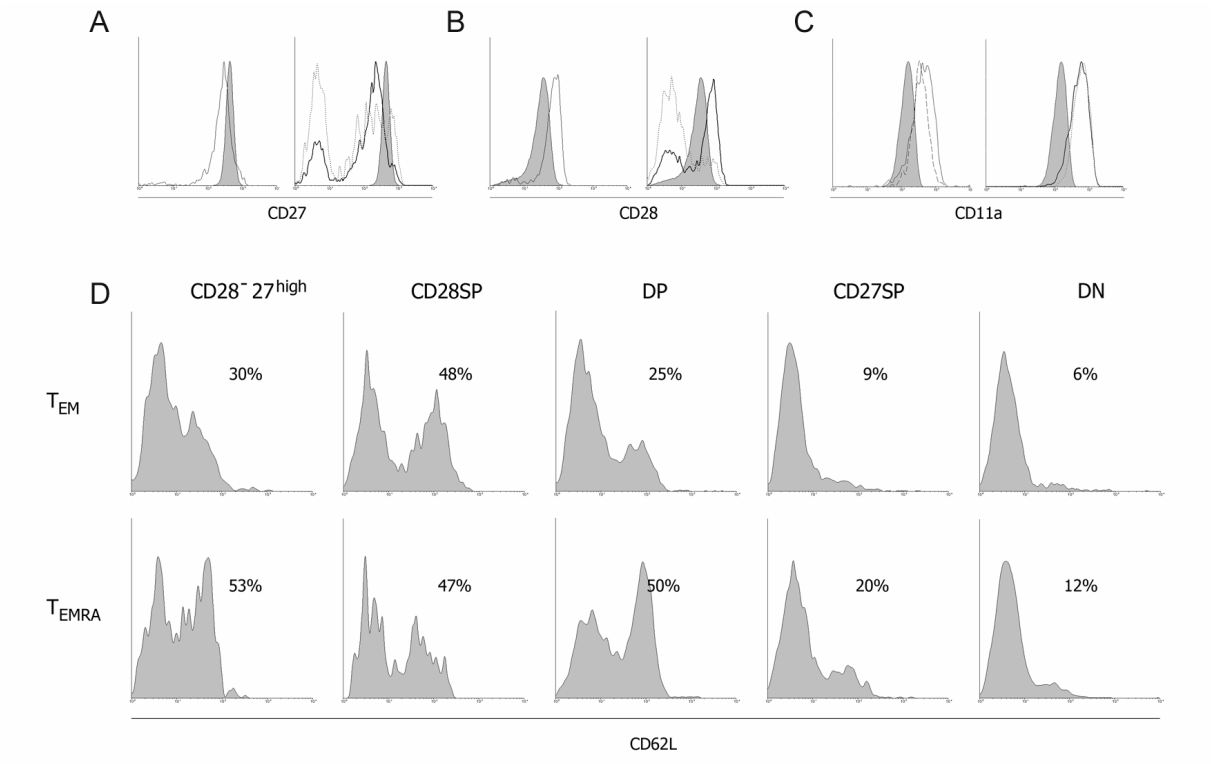


Figure 1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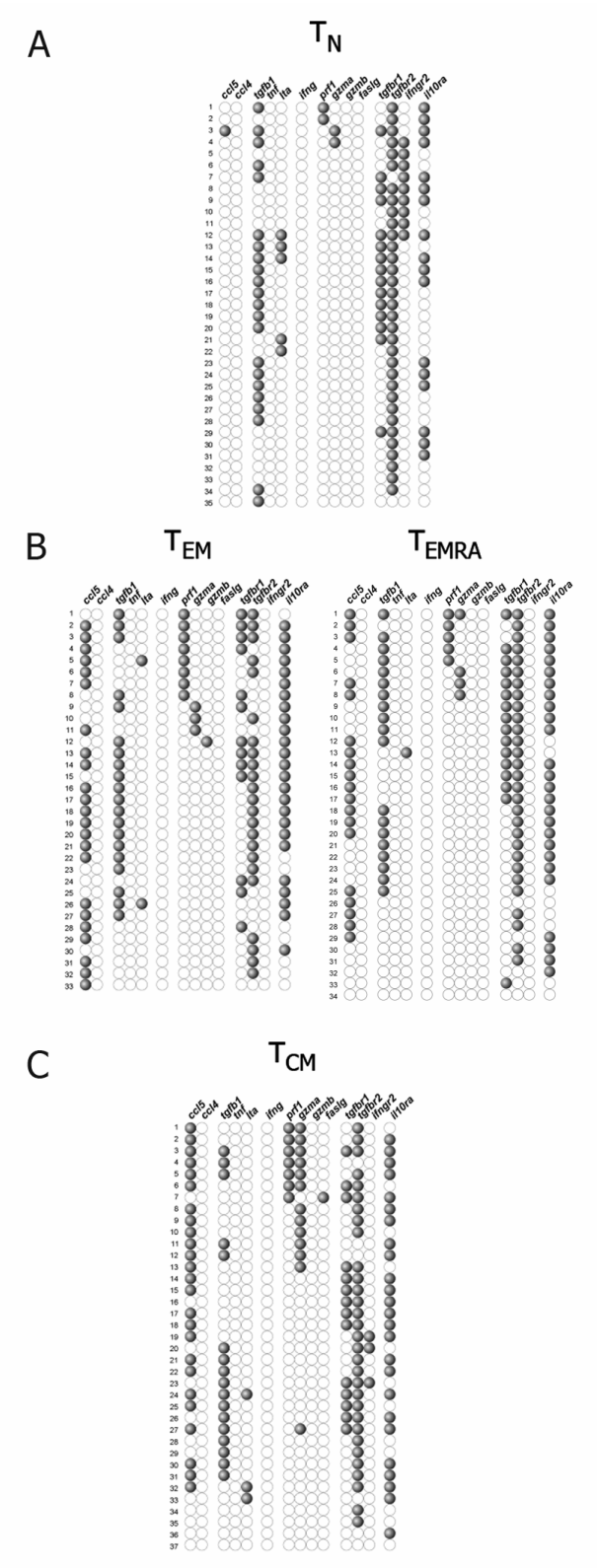
12%
CD62L

Figure 2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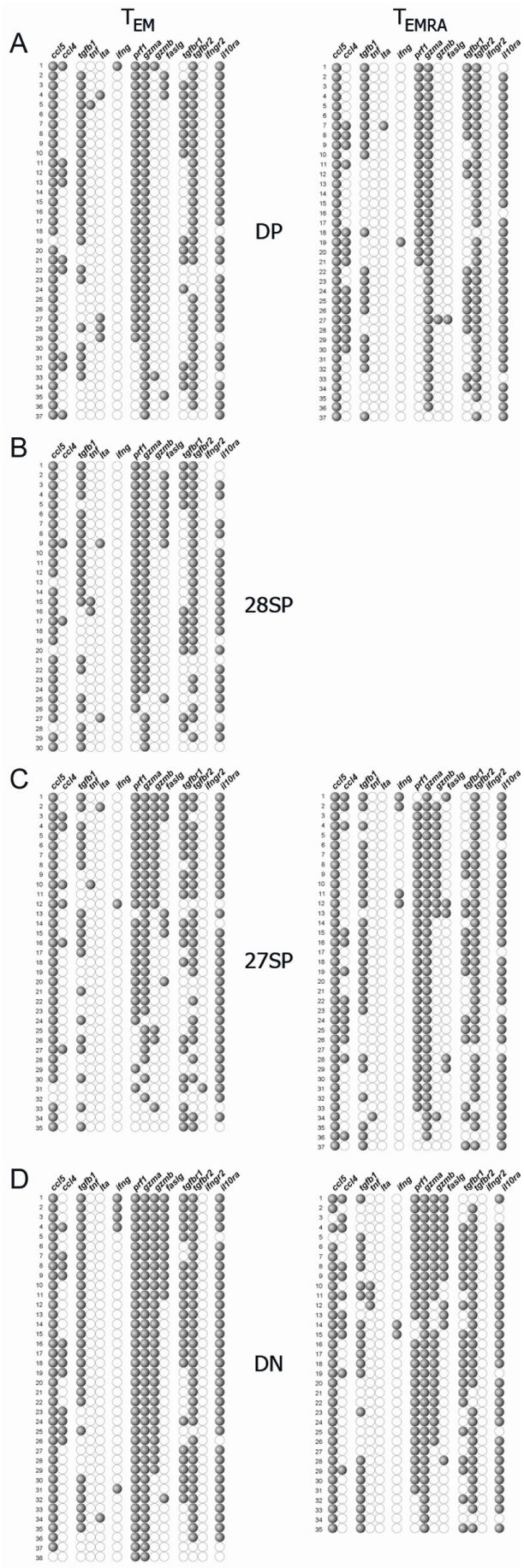
T_{EMRA}
C
T_{CM}

Figure 3

Figure 4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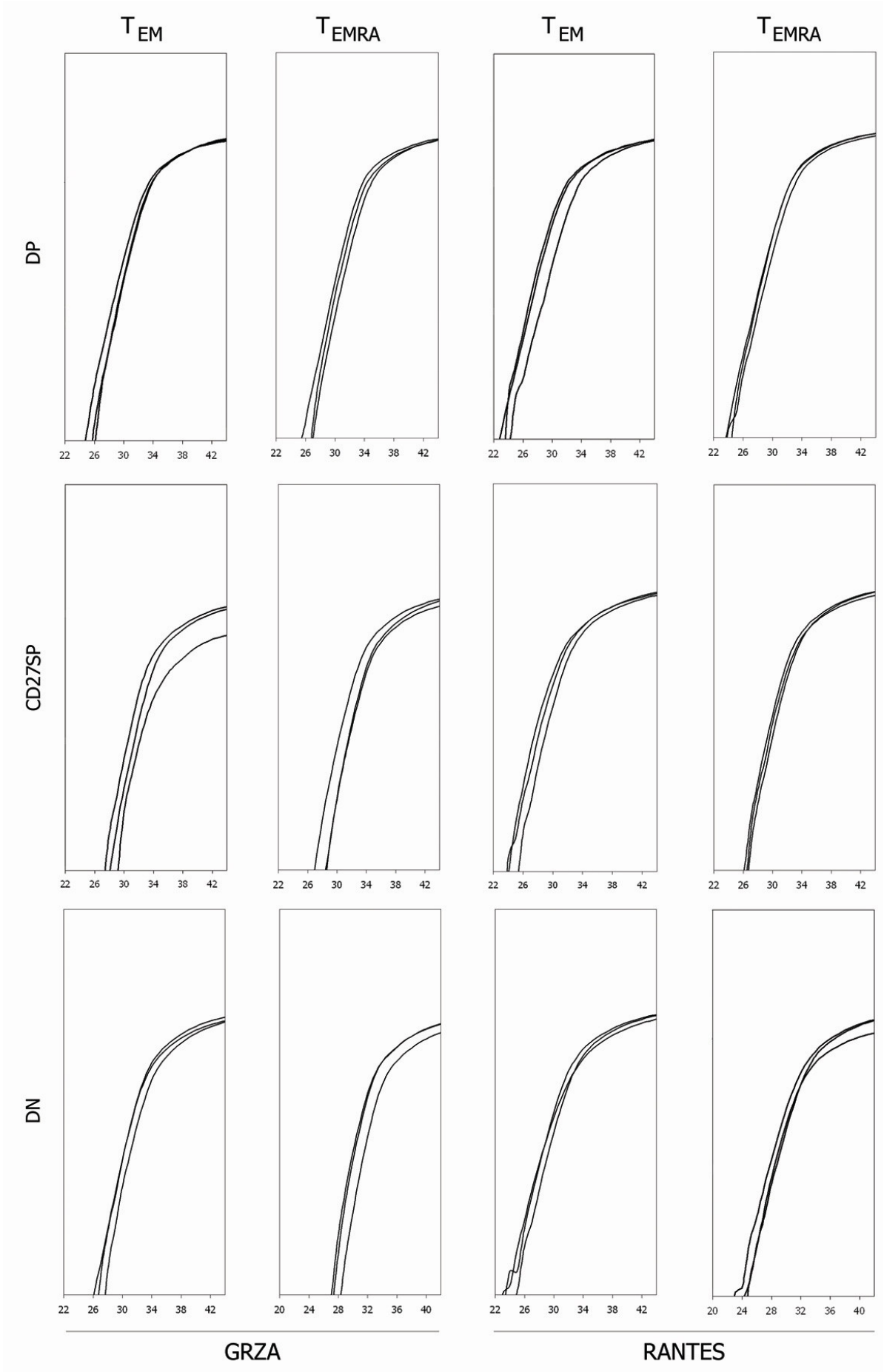
tgfbr2
ifngr2
il10ra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T EMRA
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CD27SP
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22 26 30 34 38 42
20 24 28 32 36 40
GRZA
RANTES

Figure 5

DISCUSSION

Part I. Single-cell multiplex RT-PCR

The heterogeneity of lymphocyte populations defines the spectrum of potential fates and roles that the individual compound cells might have. Cell fate and function are actually deeply conditioned by different patterns of gene expression. Hence, the study of the cellular gene expression balance is crucial to understand several types of biological events and differentiation processes, and has a major impact on the comprehension of the behavior of cellular populations.

Different cell fates likely rely on both qualitative and quantitative differences of gene expression affecting multiple genes. For example, in the mouse it has been demonstrated that resting memory T cells maintain certain functions after antigen elimination, which suggests that differences between memory and effector cells may be more quantitative than qualitative (Opferman et al., 1999; Selin and Welsh, 1997; Veiga-Fernandes et al., 2000). Additionally, acquisition of effector functions may require the co-expression of several effector molecules by the same cell (e.g., co-expression of perforin and granzymes for effective cytotoxic activity) and this information is fundamental on the evaluation of vaccination protocols that may rely on the induction of such differentiation programs. However, methodologies available for studying gene expression present several limitations. Micro-arrays and RT-PCR, which allow the detection of multiple gene expression, only enable assessments at a population level. In turn, techniques that permit the study of multiple gene expression on single-cells are just qualitative and allow studies of only few genes. Furthermore, *in vitro* studies were shown to induce modifications in the cellular behavior. In particular, the capacity of T cells to proliferate *in vitro* does not correlate with their capacity do proliferate *in vivo* (Bachmann et al., 1999a; Tanchot et al., 1998; Veiga-Fernandes et al., 2000), and the production of cytokines by individual cells, upon short-term *in vitro* reactivation, is extended to a higher frequency of cytokine secreting cells (Panus et al., 2000; Veiga-Fernandes et al., 2000). New approaches allowing the study of the gene expression of individual cells directly *ex vivo* and in a quantitative fashion were therefore lacking.

We developed a new technique of quantitative single-cell multiplex RT-PCR that overcomes all the previous limitations. This method allows the study of

individual cells directly *ex vivo* and the simultaneous quantification of a vast array of cellular transcripts (up to 20) at a single-cell level. Since it can be performed with very small numbers of cells up to one cell, it has a significant interest for the analysis of minute samples, which commonly are the only source of diagnosis in several pathologies in humans. Furthermore, it is a very sensitive technique allowing the detection of 2 molecules per cell. As several genes are amplified simultaneously, competition between different primers or for limiting resources were common limitations for the simultaneous study of multiple cell functions. We show that in our method, despite the simultaneous amplification of several molecules, all PCRs have the same efficiency. In addition, this method also enables the progress from quantitative assays performed at population level to quantitative assays at single-cell level. This allows discriminating if a raise or diminution on the expression of a given function, such as cytokine production, is due to a variable frequency of cells expressing that function or, instead, results from an increased or lower amount of function produced per cell.

An additional advantage of this method is the possibility to quantify the absolute number of transcripts present in a cell, in contrast to the classical real-time PCRs that in reality only provide a relative estimation of the number of messages. We used two approaches to achieve this goal. First, by using different numbers of cells, we established control curves correspondent to different copy numbers based on the amplification of the genomic DNA. Since each cell has two copies of DNA coding for each gene, we could run a scale based on a range of cells in parallel with our RT-PCRs and from these curves of amplification estimate copy numbers. Secondly, both the analysis and the precise quantification of the absolute copy number of messages present in a cell depend not only on the PCR efficiency, but also on the efficiency of the reverse transcription reaction. We addressed the later point by comparing the amplification of a known number of mRNA molecules of a given sequence (where the RT reaction precedes the amplification), with the amplification of an identical number of double-stranded molecules of the same sequence (where the RT reaction is not required for the amplification). This strategy enabled us to precisely determine the efficiency of the reverse transcription step in our method. In addition, the synthesized double-stranded molecules allowed us to establish a standard scale for PCR quantification that avoids the inconvenient manipulations needed for sorting different cell numbers required for the DNA calibration.

This method brings new perspectives for the study of lymphocyte populations *ex vivo*. Hereafter, it is possible to accurately determine the kinetics of expression of distinct cellular functions in multiple immunological events, such as primary and secondary immune responses or hematopoiesis. Although these issues can be partially addressed by techniques using a population approach, the exact evaluation of both frequency of cells expressing given functions and the amount of each function produced in a per cell basis, can only by achieved by means of a quantitative single cell method. Furthermore, this method can be applied in the study of different species, in particular mice and men. Finally, it can also be adapted to study the expression of a flexible set of genes. Indeed, this approach is currently being used in the study of different aspects of the murine and human hematopoiesis, in the study of the immune response to the challenge of different pathogens in the mouse system, and in the characterization of the lymphocyte populations isolated from human secondary lymphoid organs, such as lymph nodes and spleen. The main conclusions attained in the study of the human $CD8^+$ T lymphoid populations isolated from the blood described on the next pages illustrate well the advantages conferred by this approach.

Part II. Characterization of human $CD8^+$ T-cell subpopulations

The $CD8^+$ T-cell compartment harbors several subpopulations with multiple functionalities, including naïve, effector and memory subsets. As previously detailed in the last section of the introduction chapter, the prevailing data concerning the description of these subsets in the human peripheral blood is rather fragmentary, since a multitude of studies used a different and limited set of surface markers to identify each subset. A comprehensive analysis of the global cell-surface pattern of co-expression of the most commonly used markers in human $CD8^+$ T cells was, thus, lacking.

The central goal of this thesis was to fulfill the gaps resulting from the multiple disconnected studies concerning the characterization of human $CD8^+$ T-cell subsets and unify the overall knowledge relating to this issue. For that purpose we analyzed the $CD8^+$ T-cell compartment by simultaneously crossing the most common and relevant cell-surface markers described in the literature, namely CCR7, CD45RA, CD27 and CD28. This approach led to the discrimination of fourteen subsets of $CD8^+$ T cells, including the minor and uncharacterized $CD27^{high}$ subpopulations. In addition, we could directly compare the primed subsets present within the $CD45RA^+$ and $CD45RA^-$ compartments. This comparison is extremely relevant, since up to now the precise differential functions between $CD45RA^+$ and $CD45RA^-$ subpopulations remained unclear and are thus vaguely referred. Finally, we choose a novel strategy to study the $CD8^+$ T-lymphocyte subpopulations: the single-cell multiplex RT-PCR. We found this approach would be very informative regarding the global gene expression of each T-cell subset and, at the same time, would be a helpful tool to assess the heterogeneity of subpopulations. In addition, despite information being restricted to the mRNA level, we found this method gives valuable and discriminating insight concerning the functional potential of each cellular subset.

Rarely detected genes: IL-10, IL-2 and MIP-1α

IL-10 is a multifunctional cytokine that regulates a variety of functions of hematopoietic cells. Its main role seems to be containment and eventual

termination of inflammatory responses, enabling elimination of infectious organisms with minimal damage to host tissues (Moore et al., 2001).

Transcription of the IL-10 gene is regulated by Sp1 and Sp3, two transcription factors expressed constitutively by many different cell types, including T cells (Tone et al., 2000). It was reported that comparable promoter activity could be measured in resting and stimulated T cell lines, but the levels of IL-10 mRNA varied after activation. Such control is achieved by degradation of IL-10 mRNA, which contains several instability elements, and thus act at the posttranscriptional level (Powell et al., 2000). This strategy may perhaps facilitate a more rapid control of IL-10 expression than can be achieved by activation of transcription (Moore et al., 2001).

In our study of gene expression by single-cell RT-PCR, expression of IL-10 was rarely detected in all of the sorted $CD8^+$ T-cell populations. If regulation of IL-10 expression in physiological conditions occurs as described for T-cell lines, $CD8^+$ T lymphocytes in the blood failed to receive the appropriate stimuli that induce posttranscriptional stabilization of IL-10 mRNA and ultimately lead to the expression of this cytokine. $CD8^+$ T lymphocytes expressing IL-10 should therefore be encountered elsewhere in the body. We have isolated $CD8^+$ T lymphocytes from human lymph nodes and we have begun the study of $CD8^+$ T-cell subpopulations by multiplex RT-PCR at the single-cell level. Our preliminary results indicate that in some $CD8^+$ T-cell subsets we could detect significant frequencies of IL-10 producing cells, up to 20% (unpublished results). These evidences strongly suggest that IL-10 is produced by $CD8^+$ T cells following activation in the peripheral lymphoid tissue. The production of IL-10 could possibly result from the high levels of co-stimulation and/or helper signals that $CD8^+$ T cells receive in these sites. Our observations do not exclude the possibility that $CD8^+$ T cells can also receive, in the sites of injury, similar stimuli that allow IL-10 secretion, which might be fundamental for the termination of some inflammatory responses occurring in those sites.

Similarly to IL-10, we failed to detect expression of IL-2 in $CD8^+$ T cells circulating in the peripheral blood. However, in some cell subsets of the lymph nodes, a modest (~15%) frequency of cells expressed the mRNA coding for IL-2. This cytokine was reported to be early expressed gene following T-cell activation (Ullman et al., 1990). Its expression is induced within hours after TCR engagement. Furthermore, the mRNA coding for IL-2 is extremely instable and

rapidly degraded in the absence additional stabilization factors, such as co-stimulation through CD28. These reasons might explain why IL-2 expressing cells could be detected exclusively in the lymph node $CD8^+$ T-cell populations.

Macrophage inflammatory protein 1-α (MIP-1α, or CCL3), similarly to RANTES and MIP-1β, is a chemokine that attracts immunocompetent cells to the sites of infection. Its production and secretion generally requires cell activation. MIP-1α shares with RANTES and MIP-1β the same receptor, CCR5, whose expression is modulated upon chemokine binding (Rossi and Zlotnik, 2000). By single-cell RT-PCR, in striking contrast to MIP-1β and especially RANTES, we detected expression of the mRNA coding for MIP-1α only sporadically. This result indicates that circulating $CD8^+$ T lymphocytes producing MIP-1α are very rare (<5%). In the mouse, MIP-1α production by T lymphocytes following activation was shown to be induced *in vitro* by co-stimulation through CD28 (Herold et al., 1997). Together with our results, this data raises the possibility that T cells expressing the transcript for this chemokine should more probably be found in the secondary lymphoid organs. Surprisingly, in our preliminary analysis of the lymph node $CD8^+$ T-cell subpopulations, we were unable to detect expression of MIP-1α as well. However, in single-cell analyses we performed on splenic $CD8^+$ T-cell subpopulations, we found within some $CCR7^-$ subsets cells expressing MIP-1α that could reach a frequency of 20% (unpublished results). These observations indicate that $CD8^+$ T lymphocytes receive the appropriate signals that induce MIP-1α expression mostly in the spleen. The nature of these signals is uncertain and our data suggest that they might be delivered by cells that are excluded from the lymph nodes, but not the spleen. In the literature, production of MIP-1α (and MIP-1β) is described to be induced by several proinflammatory stimuli, including LPS, viral infection, TNFα, IFNγ and others (Maurer and von Stebut, 2004). Interestingly, we found high frequencies (>50%) of cells expressing TNF-α and IFN-γ in the spleen, but not in the peripheral blood or lymph nodes. Thus, induction of MIP-1α expression by $CD8^+$ T cells may require TNF-α and/or IFN-γ signals, whereas, in contrast, these signals are apparently not essential for expression of the highly related chemokines MIP-1α and especially RANTES (discussed later). Another possibility is that MIP-1α synthesis by human $CD8^+$ T lymphocytes occurs preferentially in the site of infection, in response to signals delivered by other cells

that were also recruited. The two hypotheses are not mutually exclusive. Alternatively, MIP-1α mRNA might be more unstable than MIP-1β and RANTES mRNAs, and TNF-α and/or IFN-γ signals are continuously required for stabilization of MIP-1α transcript. This hypothesis seems unlikely since MIP-1α and MIP-1β are highly related chemokines sharing 57% of the gene sequence, and up to now no differential regulation of gene expression was reported.

Our observation that MIP-1α is expressed by $CD8^+$ T lymphocytes in the spleen is in line with previous reports ascribing to MIP-1α an important role on the differentiation of $CD4^+$ T lymphocytes into T_H1 polarized cells. It has been described that MIP-1 proteins can regulate immune responses by modulating T_H differentiation (Rossi and Zlotnik, 2000). Consistent with this possibility is the fact that CCR5-deficient mice display T_H2-skewed cytokine profiles (Andres et al., 2000).

The fact that MIP-1α, but not MIP-1β, expression is virtually absent from the human peripheral blood, together with the observation the incidence of cells expressing MIP-1β is always superior to the frequency of MIP-1α-expressing cells in the spleen (unpublished results), strongly suggests a functional specialization. As mentioned above, MIP-1α, similarly to RANTES and MIP-1β, bind CCR5, a chemokine receptor expressed mostly by macrophages and to lesser extent by activated T cells and DCs. In addition, MIP-1α and RANTES, but not MIP-1β, can also bind CCR1, which is mostly expressed by macrophages and T cells, and less significantly by neutrophils and eosinophils (Maurer and von Stebut, 2004). Interaction of MIP-1 proteins (and RANTES) with their cognate receptors is known to be crucial in the orchestration of acute and chronic inflammatory responses, mainly by recruiting cells for the sites of infection, including T cells. The fact that MIP-1β can not bind CCR1 implies that only MIP-1α and RANTES can act on cells expressing CCR1. Collectively, these data together with our results suggest that both MIP-1α and MIP-1β are very efficient in attracting macrophages, which express high levels of CCR1 and CCR5. Nevertheless, since a higher proportion of cells expressed MIP-1β, this chemokine should play a major role in the recruitment of macrophages by $CD8^+$ T lymphocytes in relation to MIP-1α. Conversely, since CCR1 is expressed in higher levels than CCR5 in T lymphocytes (Maurer and von Stebut, 2004), MIP-1α might be playing a prominent

role in inducing chemotaxis on T cells. Nevertheless, a significant role for RANTES can not be excluded.

Expression of cytokines by CD8$^+$ T-cell subsets

TGF-β1

As discussed previously, TGF-β1 is a multifunctional cytokine whose spectrum of action depends of several external factors, such as co-stimulation, cytokine context, etc. Expression of TGF-β1 in an important fraction of cells was detected in all the CD8$^+$ T-cell subpopulations studied by single-cell RT-PCR. Nevertheless, no drastic differences of TGF-β1 expression were observed between CD8$^+$ T-cell subpopulations, apart perhaps a modest tendency of slightly higher frequencies by more differentiated CCR7$^-$ subsets (DP, 28SP, 27SP and DN), as compared to CCR7$^+$ and CD27^{high} cells. CCR7$^-$ CD8$^+$ T-cell populations with distinct degrees of differentiation, defined by differential expression of CD27 and CD28 molecules, contain comparable frequencies of cells expressing the mRNA coding for this cytokine. Furthermore, no pattern of segregation with other particular functions, such as its own receptors, could be ascribed. For these reasons, it is difficult to attribute a specific significance or specify a particular function to the expression of TGF-β1 in the studied populations, at this stage.

TNF-β

TNF-β, also named lymphotoxin α (LTA), is a cytokine expressed prominently in the lymphocyte compartment. When produced as homotrimers TNF-β is secreted, but it can also be found in a membrane bound form when complexed with monomers of another related protein, lymphotoxin β. Signaling initiated upon ligation of TNF-β to its receptor is thought to be crucial for the induction of key cytokines, chemokines and other factors that organize and maintain the complex microenvironment within lymphoid tissues (Fu and Chaplin,

1999; Ware et al., 1995). A role in lymphoid organ development was also reported. TNF-β can also act in the periphery, presumably as a modulator in the immune response. In particular, it is considered to be a key element in promoting inflammatory processes (Ware, 2005).

Recent studies in the mouse system revealed that TNF-β has a constitutive expression on naïve lymphocytes within lymphoid organs, perhaps contributing to the homeostasis of the tissue. Antigen-specific and nonspecific activation of T lymphocytes from peripheral blood can also induce transcription and protein expression of TNF-β. Cytokines, like IL-2, seem to play an important role in the regulation of TNF-β expression by human T lymphocytes (review by Ware, 2005).

The expression of TNF-β on single-cells studied by RT-PCR was extremely inconsistent between populations and donors. For instance, it is consistently expressed by 15% of the naïve $CD8^+$ T cells in two donors, but absent from a third one; or, in one donor only $CD27^{high}$ and T_{CM} contain some cells expressing TNF-β, while in another cells expressing the mRNA coding for this cytokine were found in nearly all populations. Moreover, quantitative assess of the number of TNF-β mRNA molecules produced per cell showed that cells from all the subsets express comparable amounts of this transcript. Collectively, these results are quite puzzling and do not indicate a clear pattern of expression that correlates with surface phenotype. It might be possible that is not associated to a genetic programming in $CD8^+$ T cells inherent to the general state of "naïve", "T_{CM}", "T_{EM}-DP", etc. If this is the case, expression of TNF-β may possibly be influenced by external factors, such as the cytokine environment, independently of the differentiation stage of the cell. Further investigation on the factors that influence TNF-β gene expression is required to confirm this hypothesis.

Expression of several types of receptors by $CD8^+$ T-cell subsets

Single-cell analysis of mRNA expression of the $CD8^+$ T-cell subsets revealed that the great majority of the cells of all the populations express the mRNA coding for several receptors, like the receptors 1 and 2 for TGF-β (TGF-

βR1 and TGF-βR2), the signaling chain of the IFN-γ receptor (IFNγ-R2) and the binding chain of the receptor for the IL-10 (IL10Rα).

TGF-βR1 & TGF-βR2

In all the studied $CD8^+$ T-cell subsets, we found a considerable expression of the receptors for the TGF-β, but no substantial differences on their expression pattern between the distinct $CD8^+$ T cell populations. Since TGF-β is a cytokine that regulates multiple cellular processes in T lymphocytes, including proliferation, differentiation and survival, such a comparable responsiveness to TGF-β by the $CD8^+$ T-cell subpopulations does not necessarily mean that this cytokine will trigger the same outcome on each one them. In particular, target cells at distinct differentiation states may receive different additional regulatory signals, including co-stimulation and cytokine signals, which can influence TGF-β regulation of T cell activity (Li et al., 2006). Therefore, the true meaning of these results is rather difficult to infer at this stage.

In the naïve compartment, our results show that, in general, TGF-βR1 was expressed simultaneously to TGF-βR2 in more than 30% of the cells. These evidences indicate that only one third of the naïve $CD8^+$ T cells can potentially be regulated by the action of TGFβ. Importantly, in naïve T cells, TGF-β was reported to inhibit antigen-driven expansion *in vitro*, in the absence of co-stimulation (Kehrl et al., 1986). The anti-proliferative effect of TGF-β is, however, decreased by CD28 co-stimulation. Furthermore, anti-proliferative effect of TGF-β apparently depends on the strength of stimulation. Hence, at weak stimulatory conditions TGF-β prevents naïve T-cell expansion at a greater extent than when stronger stimuli are provided (Gunnlaugsdottir et al., 2005). Cottrez and co-workers have proposed a model for regulation of TGF-β responsiveness by T cells during activation (Cottrez and Groux, 2001). This model predicts that naïve T lymphocytes generally express TGF-βR2, which would render them susceptible to negative regulation by TGF-β, thus preventing inappropriate activation by antigens presented by nonprofessional APCs. Upon stimulation by DCs in lymph nodes, T cells would down-regulate TGF-βR expression and migrate to sites of infection without being negatively regulated by TGF-β, which is widely expressed in different tissues. Our results are in disagreement with this hypothesis. Despite

circulating naïve $CD8^+$ T lymphocytes widely express TGF-βR2, co-expression of TGF-βR1 and TGF-βR2, which determines responsiveness to TGF-β, is limited to a minor subset of cells. Alternatively, our results suggest that, *in vivo*, naïve T cells from the human peripheral blood display different susceptibilities to TGF-β regulation. The absence of TGF-β receptor co-expression on a significant subset of naïve $CD8^+$ T cells might be an indicator that these cells escape the control by TGF-β and, thus, can be activated by weaker stimuli. Only a smaller subset would require higher signal strength conditions (in terms of TCR activation and co-stimulation) in order to initiate a productive immune response. Because incubation with TGF-β prevents the expression of molecules required for execution of effector functions, but, at least in the mouse system, does not inhibit the up-regulation of molecules that usually report the activation status of the cell, Gorelik and Flavell suggested that activation of naïve T cells in the presence of TGF-β might favor the differentiation of T_{CM} (Gorelik and Flavell, 2002). Although this T-cell subset displays several typical features that fit into the classical definition of memory cell, such as long-living and enhanced proliferative capacity as compared to naïve cells, it can not exert effecter functions immediately on activation. In addition, T_{CM} were reported to be able to differentiate into T_{EM} or T_{EMRA} (Geginat et al., 2003; Lanzavecchia and Sallusto, 2000; Wherry et al., 2003). Interestingly, T lymphocytes activated in the presence of TGF-β (in the absence of IL-2) remain in a similar undifferentiated state, expressing no effector functions, and also retain a pluripotent differentiation capacity (Gorelik and Flavell, 2002). A differential responsiveness to TGF-β within the naïve $CD8^+$ T-cell compartment could, thus, reveal a division on the possible outcomes following activation: the lymphocytes co-expressing TGF-β receptors would preferentially differentiate into T_{CM}, while those lacking co-expression of both TGF-β receptors would directly differentiate into T_{EM} or T_{EMRA}. Further investigation is required to confirm this hypothesis.

A slight trend for an increased responsiveness to TGF-β, given by the co-expression of TGF-βR1 and TGF-βR2, could be ascribed to the $CCR7^-$ $CD8^+$ T-cell subsets, with a modest tendency to augment from DP and CD28SP → CD27SP → DN subsets. This might be related with the ability of TGF-β of inhibiting T-cell proliferation. However, T_N and T_{CM}, which were described to have a strong proliferative potential, are also responsive to TGF-β at a significant extent. It seems therefore unlikely that the loss of proliferative capacity might result

exclusively from the action of TGF-β. Rather, signaling through the co-stimulatory receptors, which are differentially loss in distinct activation stages, probably play a major role in this setting.

IFN-γR2

IFN-γR2 is a fundamental component of the IFN-γ R complex that plays only a minor role in ligand binding, but is required for signaling (for a review, see Stark et al., 1998). Unlike IFN-γR1, which is moderately expressed at the surface in nearly all cells, IFN-γR2 is constitutively expressed at extremely low levels. Expression of IFN-γR2 is controlled by external stimuli and its regulation is a critical factor in determining IFN-γ responsiveness (Bach et al., 1997). The best defined effects of IFN-γ on $CD8^+$ T lymphocytes are indirect and related to the increase of antigen presentation, by induction of expression of the immunoproteasome, the TAP transporter proteins and MHC class I molecules (Stark et al., 1998). More recently, it has been reported that IFN-γ can also have a direct action on $CD8^+$ T cell activity. In the mouse models of infection with *Listeria monocytogenes* and LCMV, IFN-γ was reported to regulate immunodominance of the responding cells (Badovinac et al., 2000; Tewari et al., 2004). In addition, comparison of simultaneous wild-type and IFN-γR1-deficient cell-responses to LCMV in experiments of dual adoptive transfer have demonstrated that IFN-γ acts directly upon $CD8^+$ T cells to increase their abundance during acute viral infection (Whitmire et al., 2005b).

Single-cell RT-PCR results show that expression of the mRNA coding for IFN-γR2 was observed in only two $CD8^+$ T-cell populations, naïve and T_{CM}, and the highest expression frequency resides in the former. A recent study in which human naïve, effector and memory $CD8^+$ T cells were separated according to the CD45RA/CD27 classification and the correspondent gene expression analyzed by microarrays reports that INF-γR2 expression was exclusive of naïve cells (Holmes et al., 2005). Our results disagree with this conclusion, since we have consistently detected the presence of transcripts coding for IFN-γR2 in a fraction of T_{CM} cells in all donors.

Remarkably, the maximal frequency of IFN-γR2-expressing cells found within the naïve $CD8^+$ T-cell subset did not exceed 25%. Since expression of IFN-γR2 dictates the capacity to respond to IFN-γ and, thus, identifies cells that likely play a pivotal role in an anti-viral response, it is surprising to conceive that IFN-γ responding cells might be present at such reduced frequencies. In the splenic $CD8^+$ T-cell subsets, we have found higher frequencies of IFN-γR2 expression. This finding suggests that signals required for the up-regulation of this receptor are probably provided preferentially in the sites where antigen presentation occurs. Moreover, the fact that expression of IFN-γR2 is reduced when $CD8^+$ T lymphocytes leave these sites may be an indicator that it is crucial for IFN-γ responsiveness to be avoided when the infectious agent is not present.

In the mouse system, it was described that expression of IFN-γR2 in $CD8^+$ T cells is transiently down-regulated at the mRNA level shortly after activation, being progressively regained by a smaller subset of memory cells after the expansion phase. The ability to phosphorylate STAT1, a molecule involved in the signaling pathway initiated by binding of IFN-γ to its receptor, was coincident with re-expression of IFN-γR2, indicating that responsiveness to IFN-γ correlates with mRNA expression of IFN-γR2 (Haring et al., 2005). $CD8^+$ T cells are known to produce themselves high amounts IFN-γ after antigen activation (Slifka et al., 1999). The authors have therefore proposed that loss of responsiveness to IFN-γ during the expansion phase might be necessary to evade the potentially apoptotic effects of IFN-γ during that phase of the immune response, in which it is important for activated cells not only survive, but also expand to sufficient numbers to clear rapidly growing pathogens. Our findings that INF-γR2 expression is absent from all the antigen-experienced subsets, but T_{CM}, suggest that IFN-γ stimulation might be deleterious for cells with poor proliferative potential, such as effector and memory cells of the T_{EM}/T_{EMRA} subsets. Alternatively, and since T_N and T_{CM} are two mostly undifferentiated populations that, following stimulation, will proliferate and further differentiate into effector and/or memory cells, signaling through the IFN-γR2 might be required to initiate the differentiation program that ultimately can lead to up-regulation of effector

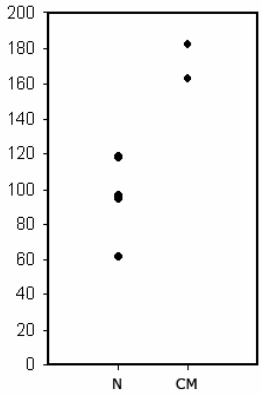

Figure 10. Number of mRNA molecules per cell coding for IFN-γR2 in naïve and central memory cells in one donor.

functions. Characterization of the putative common downstream molecules that mediate IFN-γ signaling and differentiation of effector functions would be helpful to elucidate this issue.

In the same aforementioned study, it was further reported that memory cells express IFN-γR2 mRNA at lower levels than naïve cells. We have evaluated quantitatively the expression of IFN-γR2 at the mRNA level in one donor and found precisely the opposite result (Figure 10). This difference can be explained considering the fact that the measurement of the number of IFN-γR2 mRNA molecules in the referred study was performed on total RNA extracted from the isolated populations. Our results in single cells show that the naïve T-cell pool contains a higher number of cells expressing the IFN-γR2 mRNA as compared to the T_{CM} pool, which probably accounts for the higher level of IFN-γR2 mRNA detected in naïve T cells by Haring and colleagues.

IL-10Rα

The IL-10 receptor is composed of two subunits, a ligand-binding subunit (IL-10Rα) and an accessory subunit for signaling (IL-10Rβ). IL-10Rα expression on T cells was reported to be down-regulated by activation at both the mRNA and protein levels. In contrast, IL-10Rβ is constitutively expressed in most cells and, in cells of the immune system, no evidence was found for significant activation-associated regulation of IL-10Rβ expression. Therefore, any stimulus activating IL-10Rα expression should suffice to render most cells responsive to IL-10 (reviewed by Moore et al., 2001).

IL-10 is generally considered a suppressive cytokine due to its inhibitory effects on macrophages and $CD4^+$ T cells. For instance, (1) IL-10 strongly inhibits cytokine production and proliferation of $CD4^+$ T cells via its down-regulatory effects on APC function; (2) IL-10 can also directly inhibit IL-2, TNF and IL-5 production; (3) IL-10 can further affect chemotaxis by modulating the expression of CXCR4. Nonetheless, on $CD8^+$ T cells IL-10 exerts stimulatory effects and induces their recruitment, cytotoxic activity, and proliferation. This fact suggests significant differences in the responses of $CD4^+$ and $CD8^+$ T cells to IL-10 (reviewed by Moore et al., 2001).

Expression of IL-10Rα at the mRNA level was detected in all $CD8^+$ T-cell populations studied. Furthermore, IL-10Rα expression is present in a considerable frequency of cells. The naïve population had consistently the lowest frequency of IL-10Rα-expressing cells (51%±14), followed by the primed-subsets with a lesser differentiation status (T_{CM}, 80%±17; T_{EM}-SP27high, 80%; T_{EMRA}-SP27high, 76%; and T_{EM}-SP28, 70%). The populations with higher frequencies of cells expressing IL-10Rα (>85%) were those with a more differentiated profile of gene expression (T_{EM}-27SP; T_{EMRA}-27SP; T_{EM}-DN; and T_{EMRA}-DN). These findings indicate a very clear tendency of cells to become progressively responsive to IL-10 during effector differentiation. The fact that IL-10Rα is generally expressed at least in up to 50% of the cells suggests an important role for IL-10-induced signaling. However, the induction of different outcomes by IL-10 on populations with a distinct degree of differentiation (for instance, naïve versus T_{EM}-DN) can not be excluded.

Gene expression profiles within the $CD8^+$ T-cell subsets

Naïve T cells

In terms of gene expression the T_N subset exhibits the least differentiated profile: absence of cytotoxic function and of the effector cytokines IFN-γ and TNF-α. Furthermore, the mRNA coding for RANTES, found in a significant fraction of all the other $CD8^+$ T-cell subsets, was detected only rarely amongst T_N cells. The hallmark of these cells was a preeminent gene expression concerning the genes coding for multiple receptors, just as if these cells were waiting for receiving external signals.

Our results show that naïve $CD8^+$ T cells, defined here by the co-expression of CCR7 and CD45RA, also co-express the co-stimulatory receptors CD27 and CD28, which is in line with observations made by others (Rufer et al., 2003; Tomiyama et al., 2004). However, CD27 and CD28 co-expression is not exclusive to the naïve pool, being observed in the T_{CM}, T_{EM} and T_{EMRA} $CD8^+$ T cell subsets. Therefore, cell surface co-expression of CD27 and CD28 alone do not discriminate any particular functional subset within the $CD8^+$ T-cell compartment.

The naïve $CD8^+$ T cells express uniformly CD28, however at a substantial lower level as compared with all the other populations. Up-regulation of the expression level of CD28 at the cell surface of $CD4^+$ and $CD8^+$ T cells after *in vitro* activation has been reported (Salazar-Fontana et al., 2001; Turka et al., 1990). *In vivo*, modulation of cell-surface expression of CD28 has been observed in $CD4^+$ T cells (Minguela et al., 1997; Salazar-Fontana et al., 2001). Our results are clear in showing that *in vivo* up-regulation of CD28 expression also occurs in $CD8^+$ T cells. Our results clearly show that the naïve subset exhibits the lowest levels of CD28 expression, as mean fluorescence intensity of the CD28 staining on naïve cells was consistently lower than in $CD8^+$ T cells from any other subset in all donors (average MFI difference of 12,5±4,7). We observed the same phenomenon in the mouse (data not shown). These results indicate that up-regulation of CD28 expression at the cell-surface occurs in consequence of T-cell activation. In accordance, the single-cell RT-PCR results show that expression of activation-related genes, such as the genes coding for cytolytic molecules and inflammatory chemokines, is virtually absent from naïve cells, but is up-regulated in populations expressing high levels of CD28. The frequency of expression of such genes is drastically increased on the $CD28^-$ $CD8^+$ T-cell subsets. These results indicate that acquisition of effector functions primarily relates to up-regulation of CD28 expression and, at later points, with loss of this receptor from the cell surface.

Conversely, density of expression of CD27 was higher in T_N cells than in T_{CM}, T_{EM}-DP, T_{EMRA}-DP, T_{EM}-CD27SP and T_{EMRA}-CD27SP subpopulations. This finding suggests that high levels of co-stimulation through CD27 may be crucial in the initial phases of the immune response. In accordance, we have shown that the $CD8^+$ T-cell subsets that display the closest gene expression profile to T_N cells and, thus, have the lowest activation status observed amongst antigen-experienced subpopulations, were subpopulations where the intensity of CD27 expression was up-regulated at the maximal levels observed.

Naïve T cells up-regulate CD27 following activation

In some donors, a subset of cells expressing a clear high level of CD27 was present and stood out from the T_{EM} and T_{EMRA} populations. This subset was mainly composed, albeit not exclusively, by $CD28^-$ cells. In some donors we were able to

isolate these cells to further analyze its profile of gene expression by RT-PCR. Surprisingly, despite their $CCR7^-$ phenotype, the profile of gene expression of T_{EM}-$CD27^{high}$ and T_{EMRA}-$CD27^{high}$ populations suggests that these cells are functionally not very different from naïve cells. The most striking difference from unprimed cells was a significant expression of RANTES, consistently detected in more than 50% of the cells. Importantly, in all the other $CD8^+$ T-cell subsets, for which the gene expression profiles indicated a higher activation status, expression of RANTES concerned nearly 100% of the cells. These findings indicate that T_{EM}-$CD27^{high}$ and T_{EMRA}-$CD27^{high}$ populations are in a subsequent stage of differentiation in relation to the T_N subset, but are less differentiated than all the remaining primed populations. This hypothesis was further supported by the staining of T_{EM}-$CD27^{high}$ and T_{EMRA}-$CD27^{high}$ populations for the α chain of the integrin LFA-1, CD11a. Staining for CD11a is widely used to distinguish naïve T lymphocytes, which express low levels of this molecule, from antigen-experienced $CD11a^{high}$-expressing T cells. The mean fluorescence intensity of CD11a staining in $CD27^{high}$ cells was significantly higher as compared to the T_N subset, corroborating the idea that $CD27^{high}$ lymphocytes have already encountered antigen. Interestingly, when compared to the other subsets of antigen-experienced cells, $CD27^{high}$ cells expressed lower levels of CD11a, suggesting that cells expressing high levels of CD27 correspond to a very early stage of differentiation.

Collectively, these findings suggest that $CD27^{high}$ cells have been recently activated and have just initiated a program of differentiation that can lead ultimately to fully maturation into cytotoxic effectors. RANTES expression is, therefore, one of the earliest effector molecules to be up-regulated, preceding the expression of cytolytic molecules. In accordance, it was previously reported that expression of RANTES mRNA is up-regulated between days 3 and 5 following stimulation with mitogen or alloantigen, a temporal window that precedes the up-regulation of perforin, granzymes A and B and granulisin *in vitro* (Ortiz et al., 1997; Song et al., 2000; Ullman et al., 1990) (Figure 11). The gene expression profile of $CD27^{high}$ populations strongly corresponds to the one described for cells on the late proliferation phase. Furthermore, all the primed $CD8^+$ T-cell subsets described below in this manuscript present a more differentiated gene-expression profile. Finally, it is noteworthy that the representation of T_{EM}-$CD27^{high}$ and T_{EMRA}-$CD27^{high}$ populations was significantly variable in the same individual over time (1,7 to 2,1

fold difference), suggesting that these populations might be transitory and differentiate into other subsets quite rapidly.

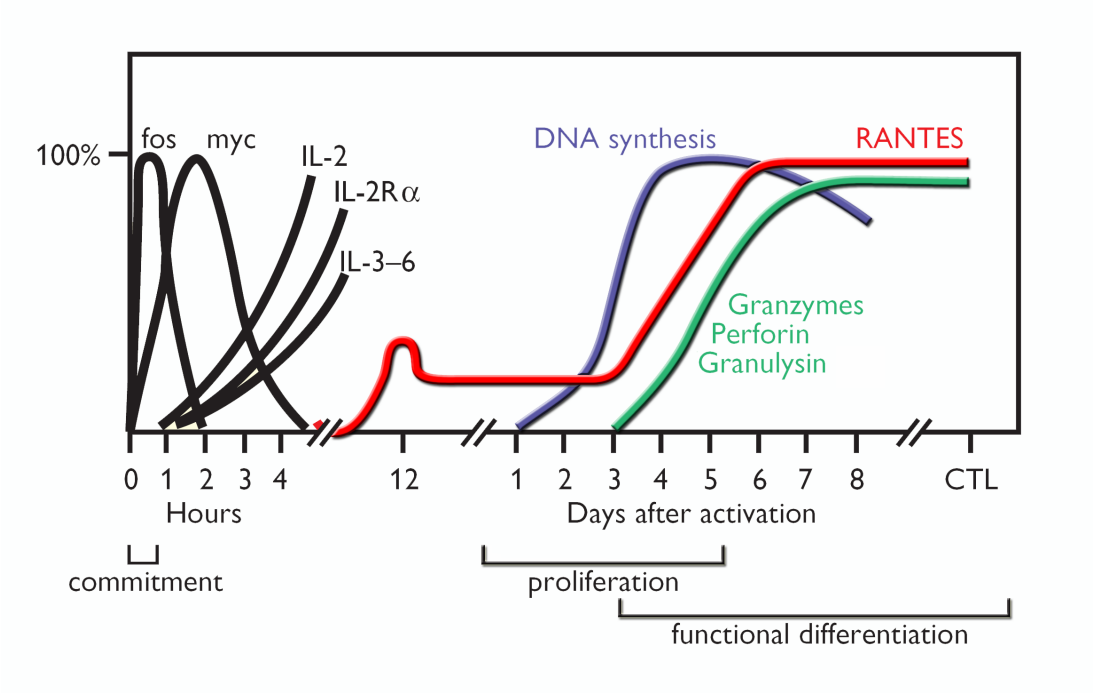

Figure 11. Sequence of gene expression events after T-cell activation by antigen. Commitment refers to the time after which withdrawal of the initial stimulus does not significantly interfere with proliferation. RANTES mRNA undergoes up-regulation on day 3-5 post-activation and its expression is maintained in terminally differentiated CTLs. Expression of the transcripts coding for proteins of the cytolytic granules was reported to be up-regulated shorter after RANTES (Figure adapted from Song et al., 2000).

Central memory CD8⁺ T cells

T_{CM} cells are described as a T-cell population with high proliferative potential, but reduced immediate effector function (Champagne et al., 2001; Sallusto et al., 1999a). Accordingly, the pattern of gene expression revealed by our results corroborates a low level of differentiation for this subset. Expression of granzyme B is absent, Fas-L expression is detected rarely and despite the mRNA coding for granzyme A could be detected in up to 40% of the cells, only a very modest percentage of T_{CM} cells (<15%) co-expresses this molecule along with

perforin. IFN-γ and TNF-α expression is consistently absent. Expression of RANTES, however, was very important and could reach more than 70% of the cells. Collectively, these findings indicate that T_{CM} possess a more activated status as compared with the $CD27^{high}$ subsets.

Interestingly, it has been described that expression of the chemokine receptor CCR4 subdivides T_{CM} cells into two distinct subsets (Geginat et al., 2003). In vitro stimulation of freshly isolated T_{CM} lymphocytes showed that $CCR4^+$ cells produce high levels of IL-4 and IL-13, while $CCR4^-$ cells produce mainly IFN-γ and, after one week of culture, down-regulate CCR7 and acquire perforin expression. These evidences led to the notion that T_{CM} cells expressing CCR4 are specialized in producing type 2 cytokines, whereas T_{CM} cells lacking CCR4 expression apparently correspond to CTL precursors. Confronting our results with these data, a reasonable hypothesis is that T_{CM} cells in which we detected the expression of cytolytic molecules might correspond to $CCR4^-$ cells or, in other words, to CTL precursors. However, since expression of type 2 cytokines was not assessed in this study, it is uncertain if the T_{CM} cells that lack cytolytic molecules correspond or not to a homogeneous $CCR4^+$ subpopulation. Further experiments would be required to confirm this possibility.

$CCR7^-$ $CD8^+$ T-cell subsets

Cells from the $CCR7^-$ $CD8^+$ T-cell subset express CD45RA in a continuum, from negative to high expression. In agreement to which is described in the literature, we divided this population into $CD45RA^+$ (T_{EMRA}) and $CD45RA^-$ (T_{EM}) and studied the surface expression of several molecules within each subset, as well as the gene expression patterns of the resulting populations. Unexpectedly, we found that the T_{EM} and T_{EMRA} subsets harbor very similar subpopulations. Expression of CD27 and CD28 subdivides both T_{EM} and T_{EMRA} subsets in a similar way, revealing the following subpopulations: $CD27^+CD28^+$ (DP), $CD27^{high}CD28^{+/-}$ ($CD27^{high}$), $CD27^-CD28^+$ (28-SP), $CD27^+CD28^-$ (27-SP) and $CD27^-CD28^-$ (DN). Some of these subsets, with the exception of $CD27^{high}$, were already detected, at least partially, by others (Appay et al., 2002; Rufer et al., 2003; Tomiyama et al., 2004). However, a direct comparison between the $CD45RA^+$ and $CD45RA^-$ was never performed, in part due to the sole partial association of the cell-surface

markers. Consequently, whether the subsets defined by CD27/CD28 expression display similar or different properties in the T_{EM} versus T_{EMRA} compartments remained illusive. The most significant difference we could find between T_{EM} and T_{EMRA} cells was on the representation of the distinct CD27/CD28 sub-populations. Hence, the DP subpopulation is usually larger on T_{EM} as compared to T_{EMRA}, the $CD27^{high}$, 27SP and CD28SP populations have comparable representations in both subsets and the DN population shows a higher frequency of cells within the T_{EMRA} subset as compared to T_{EM}.

Since the analysis of gene expression on single-cells revealed no differences between T_{EM} and T_{EMRA} populations, for simplicity I'll next simply describe the results referring to the subpopulations DP, 28-SP, 27-SP and DN.

CD27 and CD28 expression defines a hierarchy of activation

The gene expression pattern of all $CCR7^-$ subpopulations, with the exception of the previously described $CD27^{high}$ subset, reveals a degree of differentiation significantly higher than the T_{CM} subset. First, all the populations integrally expressed RANTES (>90%) and expression of an additional inflammatory chemokine, MIP-1β, was present in all populations at variable frequencies (ranging from 5 to 50%). Second, all the populations express perforin, granzyme A and Fas-L. The frequency of expression of these molecules strongly correlates with the cell-surface phenotype. Lastly, molecules like granzyme B, IFN-γ and TNF-α could also be detected, albeit only in some cell subsets.

DP subsets

The gene expression patterns of $CCR7^-$ populations revealed that the less differentiated cells were those co-expressing CD27 and CD28. Granzyme A was virtually expressed by all cells and perforin was expressed in frequencies ranging from 50 to 80%. In contrast, granzyme B expression is detected in only very few cells (<10%). In addition, expression of Fas-L is detected in up to 20% of the cells. These results indicate that (1) more than half the cells from the DP subset have cytotoxic potential, (2) both perforin/granzymes and Fas-L cytotoxic pathways can potentially be used kill, and (3) cytotoxicity exerted by the perforin/granzymes pathway can use perforin and granzyme A, but not granzyme B. The effector

cytokines IFN-γ and TNF-α were expressed only rarely by cells from the DP subset, indicating that these cells are potentially poor cytokine producers. Therefore, DP CCR7- CD8+ T cells constitute a population of cytotoxic cells that exerts its function mainly, but not exclusively, through the perforin/granzyme A pathway.

CD28SP subsets

The population with the profile of gene expression more related to the DP subset was the CD28SP. These populations are quite rare and, thus, we were able to recover cells from this subset in only one donor. Further analysis of cells with the same surface phenotype would thus be required to confirm results described in this study.

The frequency of cells co-expressing perforin and granzyme A is superior to the DP subset, as well as cells expressing the mRNA coding for FasL. However, we could not detect expression of granzyme B and frequency of MIP-1β expression is significantly lower in this subset as compared to DP cells. The reasons that might explain these results are uncertain and are may be related to the origin of the CD28SP cell subset. These cells remain poorly characterized since they constitute minor subsets of T_{EM} and T_{EMRA}, which imposes a limitation for the most common assays that usually require a significant number of cells. In studies where $CD8^+$ T cells specific for human viruses were stained for CD27 and CD28, this subset comprised always less than 5% of the tetramer specific cells, and most of the times were completely absent (Appay et al., 2002; Rufer et al., 2003). This raises the question whether the differentiation of these cells are preferentially induced by agents other than viruses. Nevertheless, whatever the stimuli that leads to their differentiation, an important fact is that CD28SP cells have lost CD27 expression, but seem to still require co-stimulation through CD28. This evidence indicates that this subset might keep the potential to proliferate, a fact that would be relevant to confirm. The loss of CD27 expression might also mean that this cell subset has a higher propensity to undergo apoptosis, since co-stimulation through CD27 is described to deliver survival signals that allow the accumulation of proliferating cells (Hendriks et al., 2003). In addition, in the mouse system, CD28 was reported to have a preponderant role in secondary challenge in the peripheral tissues, as compared to the primary immune response (Hendriks et

al., 2003). Unlike the primary response, the absence of CD28 can not be compensated by CD27 in these sites. In line with these evidences, a very likely possibility is that the CD28SP subpopulation in humans differentiates from the T_{CM} pool. The CD28SP subset would therefore harbor cells that are responding to a secondary challenge. If this is true and T_{CM} contain the precursors of CD28SP cells, differentiation might be direct from T_{CM} to CD28SP if both CCR7 and CD27 are down-regulated in concert or, alternatively, might involve intermediate stages, such as loss of CCR7, passing throughout a DP stage, followed by down-modulation of CD27 from the surface. It seems unlikely that up-regulation of CD27 might happen during differentiation from the T_{CM} pool, since this memory subset expresses RANTES at nearly 100% and display some cytotoxic potential, and up-regulation of CD27 would therefore imply a partial loss of these functions and a reversion into a less activated stage. If this scenario is confirmed, then CD28SP cells might constitute a subset with simultaneous proliferative and effector capacity. Such circumstances fit into the picture of a transitory subset that appears during the expansion phase of a secondary immune response, which could explain the rarity of cells with these phenotype. As memory cells are highly effective in eliminating antigen, it is thus probable that CD28SP will further differentiate into a more mature state, in which a large panel of effector functions is up-regulated. As described further in this manuscript, this situation correlates with loss of CD28 and acquisition of a DN phenotype. Confirmation of this hypothesis would require an evaluation of the cycling state of CD28SP cells, as well as the assessment of their differentiation potential.

The evidences presented in this work, however, do not permit to exclude that CD28SP can also arise during the primary immune response, corresponding to a transitory intermediate stage on the differentiation pathway from a naïve to a fully mature effector, DN phenotype. The CD28SP stage would be therefore an alternative pathway to CD27SP, for some cells to differentiate into DN cells. It seems, however, unlikely that CD28SP cells might arise from CD27SPs.

Another possibility is that CD28SP cells are still expressing CD27, but the extracellular domain of this receptor was cleaved and can no longer be recognized by the antibody. In this way, they could display a cell-surface “CD28SP” phenotype, but in reality being a modified DP population. Remarkably, DP and CD28SP subsets do not have very distant gene expression profiles. Nevertheless, this possibility remains to be confirmed.

CD27SP subsets

Loss of cell surface expression of CD28 correlates with a gene expression pattern that evidences a further differentiation stage than the DP or CD28SP subset. Indeed, in the CD27SP subsets there is a generalized expression of perforin and granzyme A. Furthermore, expression of granzyme B is up-regulated into important levels (ranging from 10 to 50% of the cells, depending of the donor), while the frequency of Fas-L expression shows a less striking increase (15 to 25% of the cells). IFN-γ and TNF-α, however, are still expressed by only few cells (<10%). The hallmark of the gene expression of CD27SP cells is therefore the acquisition of granzyme B expression, concurrently with a high level of perforin and granzyme A co-expression. Similarly to DP cells, CD27SP can potentially exert cytotoxicity by both perforin/granzymes and Fas-L pathways, but in addition to granzyme A, CD27SP also express granzyme B, which should account for a better capacity to efficiently respond to a broader spectrum of challenges. Therefore, DP, CD28SP and CD27SP $CCR7^-$ $CD8^+$ T-cell subsets are effector populations specialized in kill their targets through the Fas-L and perforin/granzymes pathways.

DN subsets

The pattern of gene expression reflecting the highest stage of differentiation corresponds to the DN $CCR7^-$ subset. These data are in agreement with other reports in which intracellular perforin staining was assessed and the cytolytic potential of DN was compared to CD27SP *ex vivo*, indicating that the DN subset possessed the highest perforin content and induced the highest target cell lysis (Appay et al., 2002; Rufer et al., 2003; Tomiyama et al., 2004). Furthermore, our results show that the highest frequency of expression of granzyme B and Fas-L also resides within the DN subset, an observation that is in line with a high content of these molecules in DN cells revealed by intracellular and surface stainings for granzyme B and Fas-L, respectively, reported by others (Rufer et al., 2003). Collectively, these data indicates that DN cells dispose of multiple alternative strategies to kill, which can explain the higher efficiency of target-cell lysis as

compared with other subpopulations. In addition, we found the highest frequency of cells expressing IFN-γ in the DN subsets, the T_{EM} showing a subtle propensity to contain more IFN-γ-expressing cells than the T_{EMRA}. Importantly, the capacity of express IFN-γ was restricted to a limited fraction of DN cells (up to 30%), a far reduced frequency as compared to the fraction capable of exerting cytotoxicity. For TNF-α the situation was even more extreme, as we were able to detect TNF-α expression only in a very restricted fraction of cells (<10%). These results indicate that the potential of secreting IFN-γ and TNF-α is limited to a reduced subset of circulating cells, but does not preclude that this situation might be inverted on the sites of infection and/or in the secondary lymphoid tissues. Actually, in $CD8^+$ T-cell lymphocytes we have isolated from the human spleen, IFN-γ and TNF-α expression could be extended, respectively, to up to 70% and 50% of the cells (unpublished data). It seems therefore possible that not only some particular subsets might be excluded from some organs, as we found in lymph nodes for some $CCR7^-$ subsets (unpublished data), but also the representation of cells with particular specializations (e.g. IFN-γ secretion) within each subset might vary between the blood, lymphoid tissues and/or sites of infection. A higher frequency of cytokine-producing cells in the lymphoid tissue might be explained by the fact that antigen presentation and lymphocyte activation occur in those sites. Therefore, the probability to find cells in advanced stages of functional maturation is higher in the secondary lymphoid organs, when compared to the blood. Moreover, since highly differentiated effector cells are endowed with receptors that enable them to follow multiple chemokine gradients that guide them to the peripheral tissues where infection is taking place, these sites will probably be also enriched in cells highly specialized for cytokine secretion. If this is true, may be the expression of chemokine receptors, such as CCR4, CCR5 or CXCR4, responsible for the recruitment of effector cells to the inflamed tissues, in addition to CCR7, CD27 and CD28 would allow to identify with exactitude the cells in charge of cytokine secretion.

Our results of single-cell gene expression thus clearly demonstrate that the cell-surface phenotype defined by CCR7, CD27 and CD28 correlates with gene expression profiles reflecting different degrees of activation of antigen-experienced $CD8^+$ T-cell populations. The hierarchy of activation status is the following: $CCR7^-CD27^{high}CD28^{+/-}$ < $CCR7^+CD27^+CD28^+$ < $CCR7^-CD27^+CD28^+$ < $CCR7^-CD27^-CD28^+$ < $CCR7^-CD27^+CD28^-$ < $CCR7^-CD27^-CD28^-$. These conclusions are in line

with a previous report, where the differentiation phenotype of viral specific $CD8^+$ T cells were shown to be ascribed to three subsets, $CD27^+CD28^+$, $CD27^+CD28^-$ and $CD27^-CD28^-$, which sequentially exhibited higher degree of activation given by the expression of cytolytic molecules (Appay et al., 2002). In contrast, our results disagree with the hypothesis proposed by Champagne and coworkers, which ascribes to T_{EMRA} the end stage of differentiation (Champagne et al., 2001). These authors suggest a linage differentiation pathway based on the expression of CCR7 and CD45RA, in which $T_N \rightarrow T_{CM} \rightarrow T_{EM} \rightarrow T_{EMRA}$. This hypothesis was proposed following the analysis of proliferation capacity *in vitro*, through stimulation of each one of the four subsets with anti-CD3 and anti-CD28 antibodies, and *ex vivo*, through intracellular staining for the Ki67 nuclear antigen, which revealed that cell division was mostly restricted to the $CCR7^+$ subsets. The authors therefore suggested that the T_N and T_{CM} populations function as precursors that seed the $CCR7^-CD8^+$ T-cell compartment. To confirm this hypothesis, the authors further assessed the differentiation potential of the T_{EM} and T_{EMRA} $CD8^+$ T-cell subsets upon *in vitro* antigen-specific stimulation. Neither population could divide, and the T_{EM} subset, but not T_{EMRA}, kept the potential of reverting to phenotypes of precursor cells. The authors suggested that T_{EMRA} cells constitute a terminally differentiated effector subpopulation that intervenes readily in case of antigen re-encounter, while precursor cells expand and replenish continuously the effector-cell pool. Our results argue against this hypothesis, demonstrating that T_{EM} and T_{EMRA} subpopulation display similar effector potentials. Indeed, we could find cells with characteristics of fully differentiated effectors within both T_{EM} and T_{EMRA} subsets and CD45RA expression did not correlate with any particular differentiation stage amongst antigen-experienced cells. The difference between T_{EM} and T_{EMRA} subsets must therefore rely on other parameters not related with effector function. For instance, intracellular staining of $CD8^+$ T-cell subsets for Bcl-2 *ex vivo* showed a decrease expression of this antiapoptotic molecule from which $T_N \rightarrow T_{CM} \rightarrow T_{EM} \rightarrow T_{EMRA}$ (Geginat et al., 2003). These data indicate that T_{EMRA} subpopulations might have a higher propensity to undergo cell death by apoptosis that their counterparts T_{EM}. It must be noted, however, that T_{EM} and T_{EMRA} populations were not subdivided into CD27/CD28 subsets. The lower Bcl-2 expression of T_{EMRA} cells, as compared to T_{EM}, could be due to a difference on the relative distribution of CD27/CD28 subsets within T_{EM} and T_{EMRA} compartments. It would be therefore interesting to evaluate Bcl-2 expression within the distinct T_{EM}

and T_{EMRA} CD27/CD28 subsets, along with other anti-apoptotic molecules. Alternatively, T_{EM} and T_{EMRA} subsets might follow distinct migration routes, an issue that could be addressed through the extensive analysis of expression for chemokine receptors in the two subsets.

Interestingly, CD27 expression is lost in concert with CD28 expression when human $CD8^+$ T cells mature into the last stage of effector differentiation. This evidence might be a sign that perhaps it is crucial for co-stimulation to be avoided at the end stages of $CD8^+$ cell maturation or, alternatively, that it is not required at this stage.

The gene expression profiles are very distinct between $CCR7^-$ populations defined by CD27 and CD28 expression. In addition, the pattern of expression of each of these phenotypes was extremely reproducible between all the donors studied. Remarkably, the expression of functions positively correlates with activation status of the different cellular subsets. In other words, when a particular molecule is expressed in a subpopulation, e.g., RANTES, its expression is always found, and usually at higher frequency, in all the subsets displaying higher degrees of activation. The inverse situation was observed for molecules whose expression is lost throughout progressive activation, namely IFN-γR2 and CCR7.

The finding that $CD45RA^+$ subpopulations have a gene expression profile identical to $CD45RA^-$ subsets indicates that CD45RA can not be used to distinguish subpopulations at different differentiation stages. It is currently considered that fully differentiated "effector cells" reside within the $CD45RA^+CD27^-$ compartment and "memory cells" are located in the $CD45RA^-CD27^+$ subset. Nevertheless, we found strong evidence that fully differentiated effectors can be likely found within both $CD45RA^+CD27^-$ and $CD45RA^-CD27^-$ subsets since these populations exhibit identical expression patterns. In the same way, we found that $CD45RA^-CD27^+$ and $CD45RA^+CD27^+$ subsets display similar functionalities, as well as $CD45RA^-CD28^{+/-}$ and $CD45RA^+CD28^{+/-}$.

Expression of CD62L within the $CCR7^-$ $CD8^+$ T-cell subsets

Unlike T_{CM} cells, which integrally express CD62L, T_{EM} and T_{EMRA} cells are reported to be heterogeneous concerning the expression of this molecule (Sallusto

et al., 1999b). Since analysis of the gene expression patterns of $CCR7^{-}$ $CD8^{+}$ T-cell subpopulations unexpectedly revealed no apparent functional differences between the T_{EM} and T_{EMRA} subsets, we have further investigated if there would be differences in the expression of CD62L that might indicate possible distinct migratory potentials. Expression of CD62L enables cells to interact with the peripheral node addressin (PNAd), which is one of the requirements for trafficking to the lymph nodes. Hence, cells expressing CD62L home preferentially to lymphoid organs, whereas cells that lack CD62L accumulate in the peripheral inflamed tissues, but are excluded from most lymphoid organs (Weninger et al., 2001).

Staining for CD62L of T_{EM} and T_{EMRA} subpopulations revealed that $CD27^{high}$, CD28SP and DP populations were biphasic in relation to CD62L expression and loss of CD28 expression from the surface was associated to an important decrease on the expression of CD62L. In accordance to the previously described evidences that $CD27^{high}$ populations include recently activated cells, T_{EM}-$CD27^{high}$ and T_{EMRA}-$CD27^{high}$ both contain in average 50% of cells expressing CD62L, the highest frequency observed amongst $CCR7^{-}$ subsets. Considering the natural variability observed between donors, $CD27^{high}$, 28SP and DP presented comparable biphasic CD62L patterns of expression fluctuating between 25 and 50% of positive cells. Conversely, T_{EM} and T_{EMRA}-SP27 show a considerable reduction of $CD62L^{+}$ cells, with frequencies generally bellow 25%. Expression of $CD62L^{+}$ cells on T_{EM} and T_{EMRA}-DN populations was even lower, registered consistently in less than 10% of the cells. Moreover, the intensity of expression of CD62L on DN cells was noticeably low, as compared with the other subsets. This evidence suggested that these cells are largely excluded from the lymph nodes, which we could confirm by surface phenotyping of cells extracted from human lymph nodes. A slight tendency of $CD45RA^{+}$ subsets to contain higher frequencies of $CD62L^{+}$ cells, in relation to their $CD45RA^{-}$ counterpart subsets was observed, but sometimes an inversion could also occur. Therefore, CD62L expression can not objectively discriminate $CD45RA^{+}$ from $CD45RA^{-}$ subpopulations, although it is differently expressed amongst the CD27/CD28 subsets. Expression for other chemokine receptors should further be assessed in order to conclude if these subsets effectively own completely overlapping migratory properties.

Interestingly, down-regulation of CD62L was largely associated with loss of CD28 expression. In accordance to the previously described single-cell RT-PCR

results, loss of CD28 marks a cellular differentiation state characterized by the co-expression of multiple effector functions. $CD28^-$ $CD8^+$ T cells are, thus, pluripotent effector-like cells, that achieve the highest differentiation stage after concurrent loss of CD27. The expression pattern of CD62L clearly indicates that 27SP and DN $CCR7^-$ $CD8^+$ T cells should be largely excluded from the lymph nodes and that exclusion, such as acquisition of effector functions, is a progressive phenomenon that emerges throughout differentiation and is accompanied by sequential loss of multiple surface molecules and concomitant acquisition of others.

Reversion of phenotype from $CD45R0^+$ to $CD45RA^+$

In addition to the similarities between $CD45RA^+$ and $CD45RA^-$ subpopulations, the co-existence of so many sub-populations within the $CCR7^-$ compartment suggests that differentiation towards a fully mature, DN stage might occur throughout multiple alternative ways. In other words, down-regulation of the various cell-surface markers might be asynchronous. In this case, naïve T cells receiving a given stimulus would preferentially acquire a $CCR7^-CD45RA^+$ phenotype, while in other circumstances, such as different strength of TCR stimulation, extent of co-stimulation and helper signals and/or cytokine context, would rather differentiate into a $CCR7^-CD45RA^-$ phenotype. This hypothesis questions, however, the paradigm of loss of CD45RA expression following activation, and further re-expression upon maturation into a terminally differentiation stage.

The notion that T cells down-regulate CD45RA following activation and comes from *in vitro* studies, in which activation of $CD45RA^+CD8^+$ T cells induced up-regulation of CD45R0 and loss of CD45RA. In turn, when stimulated, $CD45R0^+CD8^+$ T cells maintained their phenotype (Akbar et al., 1988). This led to the idea that CD45R0 does not only identify previously activated cells, but is also expressed in cells that have been recently stimulated. Other studies, using polyclonal T cells and T cell lines stimulated with PHA or anti-CD3 antibody, further reported that reversion from CD45R0 to CD45RA might be possible (Fujii et al., 1992; Michie et al., 1992). This issue has also been addressed *in vivo* through analysis of antigen specific cells for persistent human viruses, commonly HCMV

and EBV. Both belong to the family of herpesvirus that can establish both lytic and latent infections and affect the great majority of the population. Briefly, following primary infection, both HCMV and EBV persist life long in a latent state in the myeloid or B-cell reservoir, respectively. The chronic infections are asymptomatic, except in cases of immunosuppression. How exactly the immune system controls the infection by these viruses at long term is not clear, but $CD8^+$ T lymphocytes seem to play a central role in this setting to prevent disease. In the early time points of acute HCMV and EBV infections, there is a prevalence of epitope-specific $CD8^+$ T cells in the $CD45R0^+$ subset on the peripheral blood. In the chronic, asymptomatic phase of both infections, however, both $CD45RA^+$ and $CD45R0^+$ $CD8^+$ T-cell subsets contain significant frequencies of cells with the same specificity (Callan et al., 1998; Wills et al., 1999). It was therefore assumed that, after the primary response, some of the clonally expanded $CD45R0^+$ viral-specific CTLs revert into a memory $CD45RA^+$ phenotype (Wills et al., 1999). Although this hypothesis became generally accepted, it should however be critically considered.

First, although the majority of the CTLs from the clone analyzed by Wills and collaborators through clonotype probing have a $CD45R0^+$ phenotype at 3 and 4 weeks, ~15-30% of the $CD8^+$ T cells from the same clone express $CD45RA^+$, which is not negligible. In the primary immune response to EBV infection, $CD8^+$ T cells also mainly express CD45R0, for both EBV lytic and latent cycle epitope-specific cells (Callan et al., 1998). Still, a frequency of 5-14% of EBV-specific cells expresses the CD45RA isoform. It is therefore possible that these $CD45RA^+$ CTLs found in early time points of the acute phase of CMV and EBV infections have differentiated directly from the naïve T-cell pool. Sequencing the clones found within $CD45RA^+$ and $CD45R0^+$ compartments would be extremely informative. Secondly, although the matrix protein pp65 of HCMV is reported as being immunodominant, no other clones were analyzed. The CTLs harbored within the $CD45RA^+$ compartment are probably subdominant clones that may be exclusive of this subset. Confirmation of this hypothesis implies that other specificities must be analyzed. In another study, the relationship between the expression of CD45 isoforms and the TCR V_β usage of CMV-specific $CD8^+$ T lymphocytes on several donors was addressed (Vargas et al., 2001). Although only the chronic infection phase was studied, it is clear that major expansions of cells expressing the same V_β elements can express CD45RA or CD45R0. More importantly, the dominance

of the CD45 phenotype is extremely variable between individuals, as in some cases the immunodominant clone is predominantly $CD45RA^+$ and in others $CD45R0^+$. In addition, the dominance of the same CD45 isoforms for the same V_β specificity was constant over time in each individual.

Taken together, these studies clearly demonstrate that in acute viral infection memory $CD8^+$ T cells are preferentially located within the CD45R0 compartment and, over time, progressively become distributed between $CD45RA^+$ and $CD45R0^+$ populations. Moreover, the extent of CD45RA or CD45R0 phenotype dominance within virus-specific $CD8^+$ T cells was shown to be highly variable between individuals. At present, there is still no direct proof that antigen-specific $CD45RA^+$ cells are actual "revertants". Hence, it can not be excluded that these cells have been activated later and/or under different conditions and arise directly from the naïve $CD8^+$ T-cell pool. The replicative history of the $CD45RA^+$ $CD8^+$ T-cell subpopulations, assessed by the measurement of TRECs (T-cell receptor excision circles), supports the idea that those cells can differentiate directly from the naïve pool and, thus, a $CD45R0^+$ stage is not necessary (Rufer et al., 2003). In contrast, *in vitro* evaluation of the differentiation potential of human $CD8^+$ T cells argues that only precursors present in the T_{CM} population, but not T_N or T_{EM}, can generate $CD45RA^+$ cells (Geginat et al., 2003). However, according to this study, $CD45RA^+$ cell-differentiation from T_{CM} occurs exclusively upon cytokine stimulation, in the absence of antigen stimulation. Such situation seems quite unphysiological, since differentiation of potent effector cells, as the ones found within the $CD45RA^+$ compartment, without the tight control of antigen stimulation, as well as a selection of the antigen specificity, would represent a threaten for the organism. Nevertheless, a role for cytokines in the differentiation of CD45RA cells can not be excluded. Further *ex-vivo* evidences would be required to clarify this point.

Since HCMV and EBV are life long persistent viruses, it is likely that viral latent peptides are continuously presented to $CD8^+$ T cells that, in this way, remain in incessant activity, thus keeping infection under control. The set of viral peptides presented to CTLs, differing between acute and chronic EBV and HCMV infections, could also possibly elicit different types of $CD8^+$ T-cell responses (Goodrum et al., 2002; Tierney et al., 1994). One hypothesis in line with this idea is that highly expressed peptides of the viral lytic cycle, presented on the acute phase of the infection might preferentially induce the generation of $CD45R0^+$ $CD8^+$

T cells, while peptides expressed during the latent phase might induce the differentiation of CD45RA-expressing $CD8^+$ T cells. This could explain the generation of a subdominant $CD45RA^+$ CTL response during primary infection, resulting from the activation with latency early expressed peptides or, alternatively, peptides that might be common to lytic and latent cycles and are expressed at a lesser extent during acute infection. At present, there are no evidences supporting this idea. However, an exhaustive examination of the phenotype of a panel of clones including subdominant viral-specificities present in acute and chronic infection would be helpful to confirm this hypothesis. In addition, full understanding of the lineage relationships between the $CD45R0^+$ and $CD45R0^+$ CD27/CD28 compounding subsets would require immunoscope analysis of the correspondent repertoire.

Finally, it has been described that human $CD45R0^+$ cells turn over more rapidly than $CD45RA^+$ *in vivo* and have a higher proliferative potential *in vitro* (Champagne et al., 2001). As discussed earlier in this manuscript, the *in vitro* cell-division capacity of the four canonical $CD8^+$ T-cell subsets defined by CCR7 and CD45RA expression was determined as follows: $T_N > T_{CM} > T_{EM} > T_{EMRA}$, being extremely reduced in T_{EM} and virtually absent in T_{EMRA}. This led to the assumption that the precursors of T_{EMRA} must be harbored within the $CCR7^+$ subsets. Although this theory does not exclude the possibility that T_N can directly differentiate into T_{EMRA}, in a secondary immune response, the responding, proliferating cells that could differentiate into T_{EMRA} would forcedly be T_{CM}, or eventually T_{EM}. In either case, this scenario implies that cells expressing CD45R0 (T_{CM} or T_{EM}) in the beginning of the immune response down-regulate that molecule and revert into a $CD45RA^+$ state (as T_{EMRA}). Tracking of the cells responding to antigen would be required to solve this question.

In summary, the reversion phenomenon by which $CD8^+$ memory cells re-express $CD45RA^+$ with concomitant downregulation of $CD45R0^+$ expression has not yet been objectively demonstrated *in vivo*. This notion should be taken into account while establishing linear relationships between the different $CD8^+$ T cell subsets.

CD45 as a major role on the regulation of the signaling induced by following TCR stimulation. In particular, it regulates the extent of phosphorylation of key signaling molecules and the distinct isoforms appear to be related with a differential activity of CD45 and with the responsiveness of T cells to diverse

stimuli. In particular, the CD45R0 isoform is considered to adopt at a great extent an inhibitory conformation, thus corresponding to a less active isoform than CD45RA. Accordingly, $CD45RA^+$ cells should be more responsive to TCR stimuli than $CD45R0^+$ T cells. Thus, differentiation of $CD8^+$ T cells into T_{EMRA} could represent an advantage in terms of sensitivity to antigen stimulation in relation to T_{EM} cells, meaning that T_{EMRA} would be able to respond to lower strengths of antigenic stimulation. Clarification of this issue would be extremely relevant in order to understand the type of memory cells that should be generated after vaccination.

Loss of expression of CCR7, CD27 and CD28 – the way to differentiation

Altogether, our results concerning antibody staining of cell-surface receptors and single-cell expression analysis of $CD8^+$ T cell subpopulations of the human blood highlighted a panel of factors relevant in the differentiation of naïve $CD8^+$ T cells into different stages of antigen-experienced effectors and/or memory. We could therefore envisage a picture of the possible lineage relationships between the different $CD8^+$ T-cell subsets (Figure 12).

Firstly, activation of naïve $CD8^+$ T cells correlates with up-regulation of both CD28 and CD27. As $CD27^{high}$ were found exclusively within the $CCR7^-$ compartment, loss of CCR7 expression should occur early after antigen encounter, preceding up-regulation of these molecules. High density of CD28 molecules at the cell surface would enable activated lymphocytes to receive stronger levels of co-stimulation than naïve cells. Since $CD28^+$ cells display a high proliferative potential, likely due to high levels of co-stimulation, this differentiation process is likely accompanied by an important clonal expansion episode, in which high numbers of antigen-specific cells are generated.

At the $CD27^{high}$ stage $CD8^+$ T cells up-regulate some molecules, especially RANTES. As few $CD27^{high}$ different cells expressed single cytolytic molecules, this stage likely precedes the up-regulations of such functions. High expression of high levels CD27 at the surface might be crucial for these cells to receive survival signals that will allow them to escape apoptosis, a phenomenon to which proliferating cells became especially susceptible. Therefore, the $CD8^+$ T

lymphocytes that achieve to be co-stimulated through CD27 receptor might have higher chances to accumulate in the body and proceed into the process of differentiation.

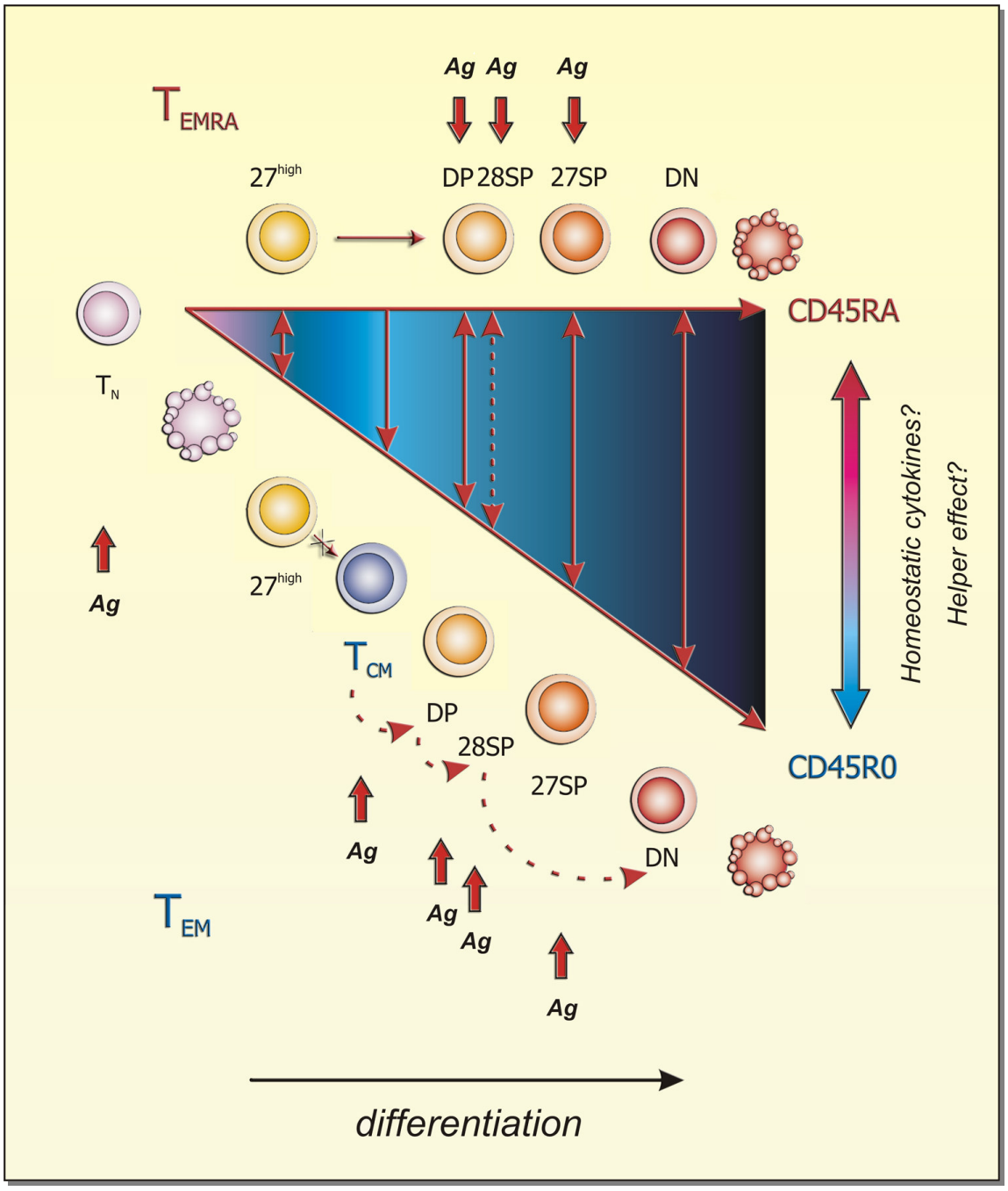

Figure 12. Hierarchy of activation stages and putative lineage relationships between CD8$^+$ T-cell subpopulations. The distinct CD8$^+$ T-cell subsets defined by the simultaneous expression of CCR7, CD45RA, CD27 and CD28 of the human peripheral blood are depicted and ordered according to the activation status. Downregulation of CD45RA expression from the surface may not be a mandatory step occurring after stimulation of naïve CD8$^+$ T cells (T_N), being influenced by factors yet undefined. Shadowed surface represents the strength of stimulation that

hypothetically is associated to the differentiation of each cellular phenotype. Excessively weak or strong stimuli induce cell death of cells. A minimal level of stimulation is believed to be needed for T_N $CD8^+$ T cells to upregulate functional genetic programs and survival factors that enable them to avoid death. Central memory (T_{CM}) cells are thought to differentiate upon low levels of stimulation that induce their fitness, but are insufficient for them to completely upregulate genetic programs associated to effector functions. Stronger stimulation is believed to generate effector memory cells (T_{EM}) and hierarchical thresholds of activation might induce distinctly differentiated subsets. The less activated T_{EM} subset expresses high levels of CD27 ($CD27^{high}$) and apparently it is transitory, giving rise to cells double positive (DP) for the expression of CD27 and CD28, single positive (SP) for one of these molecules (28SP or 27SP) or double negative (DN). Although $CD27^{high}$ cells are less activated than T_{CM}, it is unlikely that T_{CM} could be generated through a $CD27^{high}$ stage. Also, re-stimulation of T_{CM} cells likely generates DN, 27SP, 28SP or DN cells directly, without reverting to a $CD27^{high}$, less activated state (dashed arrows). The presence of $CD27^{high}$ subsets, with characteristics of recent activation, in both T_{EM} and T_{EMRA} is a strong argument for the maintenance of CD45RA expression after priming of naïve cells. Since all primed subsets but $CD27^{high}$ cells are stable populations, all of them can potentially re-encounter antigen (Ag) and elicit a secondary immune response, eventually differentiating into other more activated cell subsets.

Once the cells have received enough signals that enable them to escape apoptosis, CD27 expression probably lowers into intermediate levels, inferior as compared to naïve cells. At this phase, expression of CD28 might have, or not, been down-regulated, an event that might depend on several factors. For instance, if cells receive a very strong stimulus via the TCR, co-stimulation through CD28 is less required and, thus, expression of this receptor may be down-regulated faster. Alternatively, an extensive and productive contact with $CD4^+$ T helper cells might also relieve $CD8^+$ T cells from the need of prolonged CD28-mediated stimulation. Hence, at this stage $CD8^+$ T lymphocytes display a DP or CD27SP condition. This means that several functions are already being expressed by the majority of the cells, such as perforin, granzyme A, IL-10Rα and RANTES. Other functions, such as MIP-β or Fas-L were induced only in a limited fraction of cells. Granzyme B, depending if the cells are DP or CD27SP, can be expressed at different extents.

If $CD8^+$ T lymphocytes received a weak stimulus, or lacked appropriate CD4 help, or were in disadvantage with other lymphocytes for the contact with APC and failed to receive enough levels of co-stimulation, CD28 might possibly be kept at the surface for longer periods than in conditions of optimal co-stimulation. In this situation, co-stimulation through CD27, which also requires cell-to-cell contact, might compensate for a sub-optimal co-stimulation through CD28 and allow these cells to survive the contraction phase of the immune response. Since stimulation

of CD27 induces its down regulation, some cells might thus acquire a CD28SP phenotype. These cells eventually accumulate enough signals that allow them to finally down-regulate CD28 expression from the surface and differentiate into DN cells.

Another possibility is that $CD8^+$ T cells achieve the highest stage of differentiation through the CD27SP pathway, when conditions favor CD27 expression to be lost after CD28's. At present, there it is not known if these cells may reacquire expression of CD27 and/or CD28. Such possibility implies that DN cells should be able to revert not only the cell-surface phenotype, but also the profile of gene expression into less differentiated stages and ascribes to the immune system a high degree of flexibility and adaptation to different settings of the same infection. Conversely, reversion not being possible, loss of CD27 and CD28 would lead to an end stage. DN lymphocytes display the highest activation status and are the major subset of $CD8^+$ T cells responding to CMV chronic infection. Thus, they probably play a crucial role on the control of this, and possibly also other infections. Since these cells can not receive survival signals through CD27 and can not proliferate in response to CD28 stimulation, they should receive other signals through distinct receptors in order to survive.

Whether $CD8^+$ T lymphocytes progress sequentially through these stages following antigen encounter directly to a terminally differentiated DN stage, or become rather arrested in intermediate stages, is still undisclosed. It seems logical that the progressive loss of expression of CCR7, together with the co-stimulatory receptors, depends on the strength of stimulation that these cells receive and, interestingly, is accompanied by a progressive functional maturation. The action of other external factors can not be excluded. For instance, priming of naïve $CD8^+$ T cells in the presence of cytokines such as IL-7 and IL-15 might favor differentiation of lymphocytes with retention of CD45RA expression. Alternatively, as this isotype form confers high responsiveness of lymphocytes to TCR stimulation, loss of CD45RA might be induced in situations of extremely efficient antigen presentation, such as when high density of APCs is available. In sum, the progression throughout a particular differentiation pathway versus another, as well as cells moving forward versus being arrested in a particular differentiation stage, are fate decisions that probably rely on a complex spectrum of stimuli for which lymphocytes will compete and, plus, are likely characteristic of each type of infection.

In some situations, activation of naïve lymphocytes upon antigen priming might be incomplete, as suggested by the model of progressive differentiation (Lanzavecchia and Sallusto, 2000). This might be case, for example, of lymphocytes that encounter antigen in the last phases of the immune response, when the number of APCs and the density of antigen they present, as well as other factors, become more limited. In such a scenario, naïve $CD8^+$ T cells receive some level of stimulation, which enable them to up-regulate CD28 and enter cell cycle. In addition, they can eventually receive survival signals through CD27 that allow the induction of anti-apoptotic molecules which account for an enhanced resistance to apoptosis. However, further signaling that would permit to finish the remodeling of genes coding for effector functions, which ultimately leads to the activation of differentiation programs, fail to occur. These expanded cells become long-lived, but undifferentiated and since they have undergone the initial activation events that have rescued them from the G_0 phase of the cell-cycle, they proliferate slowly in response to homeostatic cytokines produced by other cells. Therefore, upon a further challenge, these cells can rapidly start to proliferate and also progress in the differentiation program they have initiated upon previous antigen encounter. These cells likely correspond to T_{CM} cells, but the possibility that a continuum of several degrees of undifferentiated cells might exist across the DP subset can not be excluded.

When T_{CM} cells re-encounter antigen and receive the appropriate stimulation that will allow them to proceed differentiation, they may follow the same pathways described above. However, up-regulation of high levels of CD27 seems unlikely, as it would imply a regression concerning the expression of effector functions that T_{CM} were already expressing. Since in secondary immune responses CD28 appears to have a paramount role that can not be compensated by CD27, in differentiation of T_{CM} CD27 expression might preferentially be lost before CD28. In this case, T_{CM} would progress into DN through a possible, but not necessary, DP stage from which CD28SP cells would arise. Nevertheless, the differentiation of T_{CM} throughout the alternative pathway DP → CD27SP → DN can not be excluded. A particular cytokine environment and/or differential priming conditions might eventually favor the re-expression of CD45RA.

In the context of several chronic viral infections, the establishment of lifelong protection, where viral replication is thought to be controlled by specific $CD8^+$ T cells, is conferred by different $CD8^+$ T cells subsets according to the type

of infection. As an example, in chronic *infections* by EBV, HIV and CMV, the $CD8^+$ T cells specific for each virus are enriched, respectively, on DP, CD27SP and DN cells. This strongly suggests that the distinct $CD8^+$ T cell subpopulations provide selective advantages for the control of each type of infection. Whether all, or only some particular subsets amongst T_{CM}, DP, CD27SP, CD28SP or DN are endowed with the gift of longevity, or rather they are selectively replenished with cells from one pool to the other, is uncertain. If expression of CD27 could provide significant survival advantage, expression of CD28 would assure that memory cells retain the capacity of interacting productively with professional antigen cells in order to receive co-stimulation in a further challenge, which might contribute significantly for the establishment of a promptly effective secondary response.

CONCLUDING REMARKS AND PERSPECTIVES

Although heterogeneity of human $CD8^+$ T cells has been extensively analyzed in bi- and tri-dimensional studies using the CCR7, CD45RA, CD27 and CD28 molecules, the partial use of these markers renders the characterization of naïve, effector and memory $CD8^+$ T-cell subsets rather fragmentary. A comprehensive analysis of the global cell-surface pattern of co-expression was thus lacking.

The aim of this thesis was to overcome the prevailing ambiguity concerning the functional properties of memory and effector $CD8^+$ T cell-subpopulations found on the human peripheral blood. We found that an innovative approach for such study could bring important insight onto the functional characterization of $CD8^+$ T-cell subsets, as well as concerning their lineage relationships. We have therefore developed a novel method of multiplex RT-PCR at single-cell level. This strategy allowed us to: (1) study the expression of a considerable number of molecules at the quantitative level in single cells; (2) assess the heterogeneity of each cellular subset; (3) associate cell-surface phenotype to gene expression profiles; and (4) characterize and understand minor populations that are not possible to study by other methods.

We have shown that $CD8^+$ T-cell subsets display distinct patterns of gene expression. A hierarchy of activation stages correlated to CD27 and CD28 co-expression, but surprisingly is independent of CD45 isotype expression. This finding strongly suggests that the loss of surface receptors might occur asynchronously following antigen stimulation, indicating that T_{EM} and T_{EMRA} subpopulations might play similar roles in the immune response *in vivo*. This possibility challenges the prevailing notion that CD45RA expression is necessarily downregulated after activation. The identification of populations with characteristics of recently activation within both T_{EM} and T_{EMRA} subsets provided strong evidence supporting that hypothesis. However, only a thorough repertoire analysis of T_{EM} and T_{EMRA} populations can help to confirm if CD45RA expression can be maintained after $CD8^+$ T-cell activation. We are presently establishing a project for the study of T_{EM} and T_{EMRA} $CD8^+$ T-cell subsets repertoire by immunoscope analysis.

We were also surprised to found that several genes reported to be significantly expressed by $CD8^+$ T cells had a very low or absent expression. This was the case, for example, of TNF-α and MIP-1α, and others. This finding

indicates that it may be extremely relevant to study the $CD8^+$ T-cell populations recovered on the secondary lymphoid tissues. Although the access to these types of biological samples is very limited, we were able to perform preliminary studies in lymph nodes and one spleen sample that have shown that indeed, in these sites, cellular subsets with the same phenotype as in the blood were enriched in cells expressing further functions, namely, cytokines and chemokines. This observation suggests that the degree of functional heterogeneity of the cellular subsets can vary between the blood and the sites where antigen presentation can occur. In particular, this work shows that heterogeneity concerning cytokine production by $CD8^+$ T lymphocytes can not be assessed in the blood, since circulating lymphocytes are poor cytokine producers. It will be therefore interesting to investigate if patterns of cytokine co-expression can be found in $CD8^+$ T cells isolated from secondary lymphoid organs. It will be further exciting to confirm which patterns of cytotoxic gene expression segregate with particular cytokines, and if differences at the quantitative level can be detected between functions expressed by cells in the blood or in the secondary lymphoid tissue.

Since differences between T_{EM} and T_{EMRA} could not be detected in what concerns effector functions, we wonder if cells present on these compartments would not drastically differ in other settings, including proliferation, survival or production of other cytokines and/or chemokines. We consider therefore the possibility of designing a relevant panel of genes, or several independent gene sets, that would allow studying these functions quantitatively and at the mRNA level, in order to investigate the extent of which the expression of these functions differs from subset to subset. Moreover, it may be pertinent to compare these results with functional experiments aiming to answer the same questions. This would provide a direct comparison between the outcomes observed at *in vitro* studies and the *ex vivo* potentialities of the cells.

Finally, the fact specific $CD8^+$ T-cells for EBV, CMV, HCV and HIV-1 show distinct predominant phenotypes is intriguing. This might result from a viral-induced blockage in CTL differentiation or, alternatively, the distinct $CD8^+$ T-cell subsets might provide selective advantages to respond and control to each specific viral infection. For this reason, we envisage a comprehensive study of the gene expression profiles of $CD8^+$ T-cell subsets specific for multiple epitopes of different virus. With this, we intend to clarify if a pattern of gene expression correlates only with cell surface phenotype or might also depend on antigen

specificity, in particular, for epitopes of the lytic and chronic phases of infection. Moreover, it will be interesting to address whether the gene expression profiles we found in this study are altered in the context of viral infection. We hope to provide significant progress on the understanding of the mechanism of disease, which might bring fundamental insight for the assessment of therapeutic strategies against such infections.

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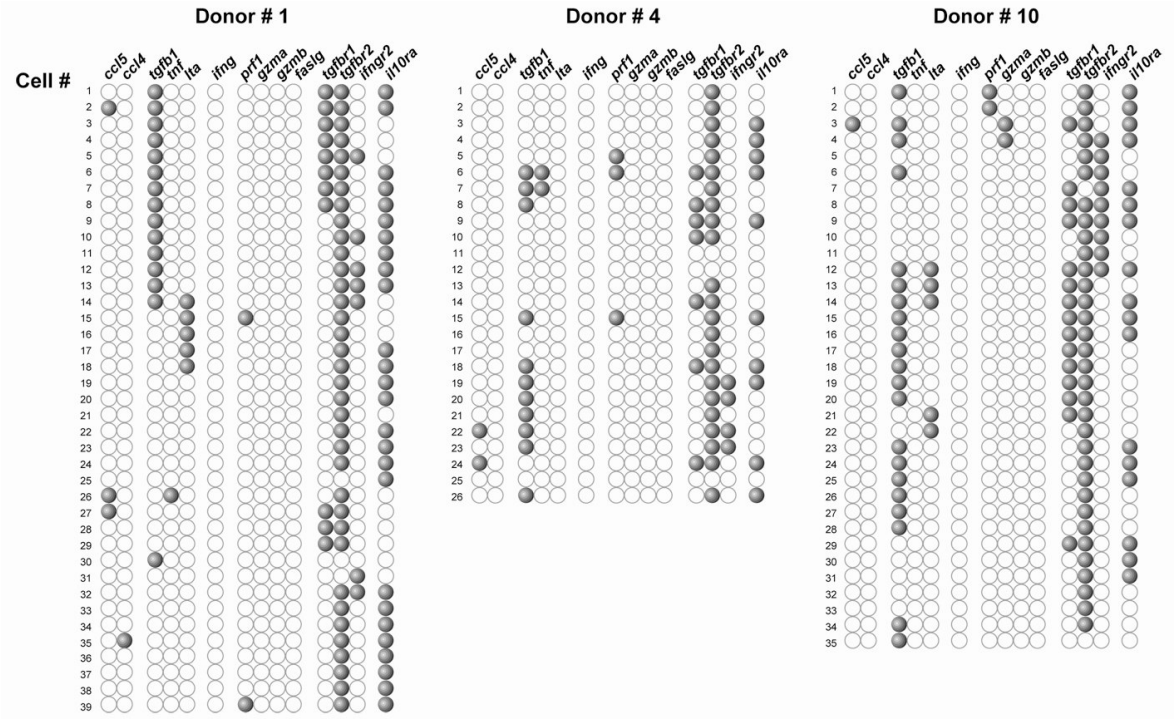
ANNEXES

Single-cell expression profiles of $CD8^+$ T-cell subpopulations isolated from the human blood

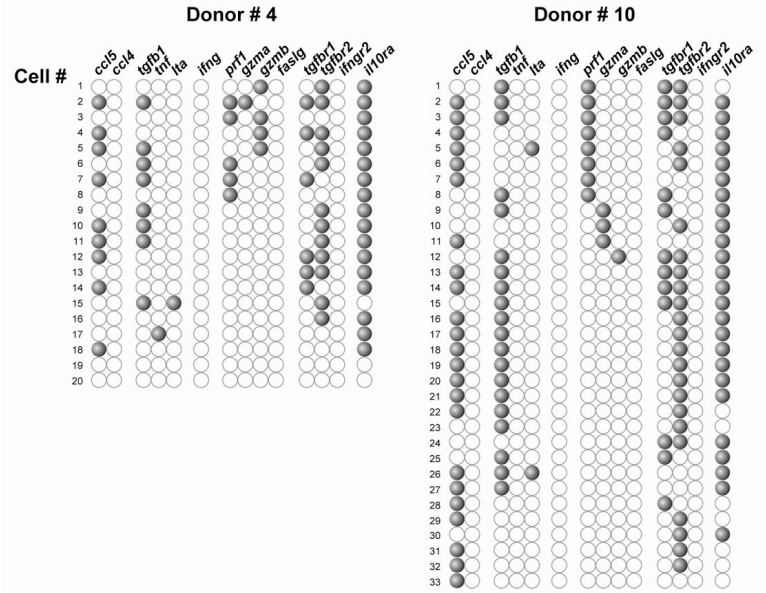
NAÏVE

Surface phenotype: $CD8^{high}$ $CCR7^{+}$ $CD45RA^{+}$ $CD27^{+}$ $CD28^{+}$



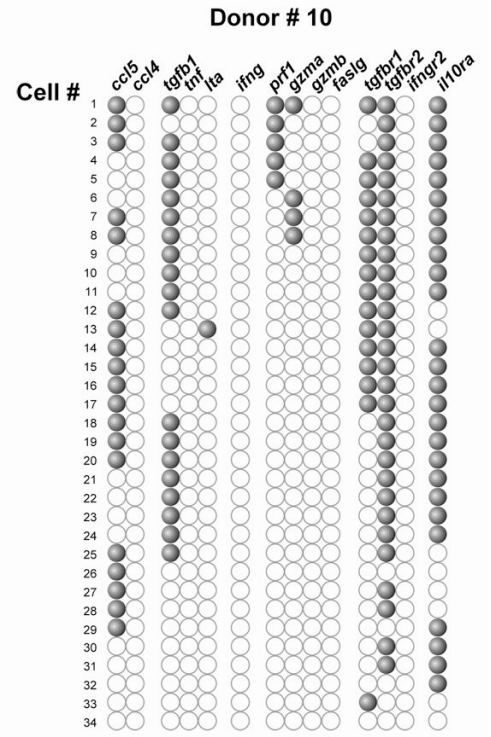
T_{EM}–CD27^{high}

Surface phenotype: CD8^{high} CCR7^{-} CD45RA^{-} CD27^{high} CD28^{-}



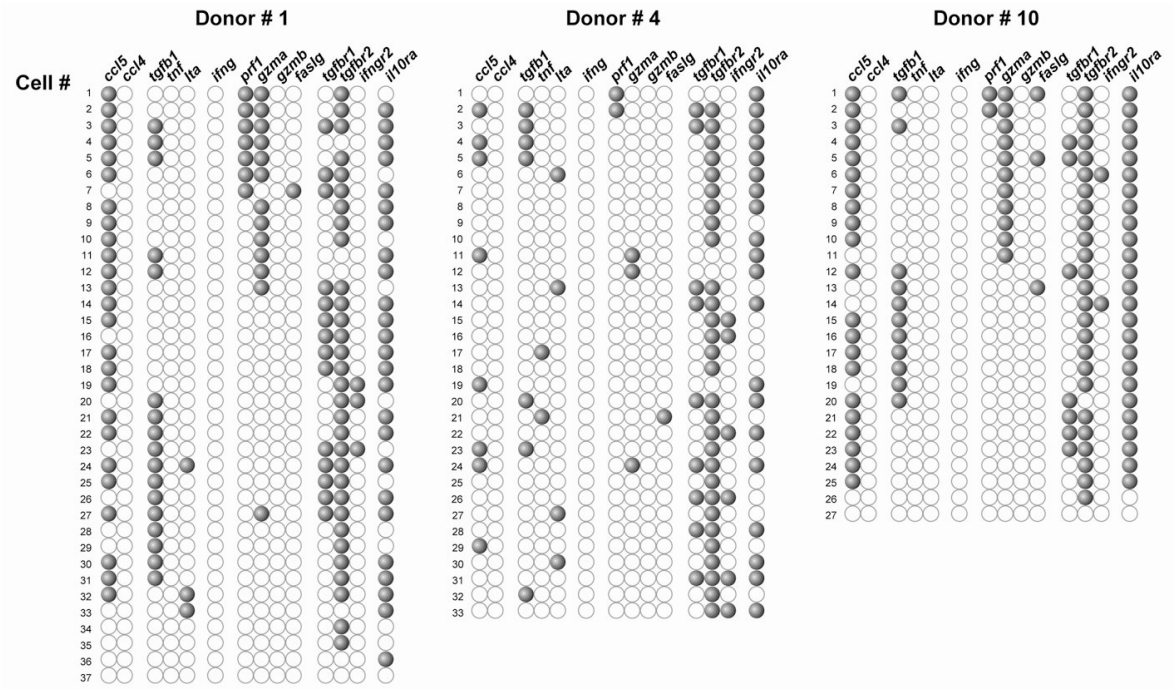
T_{EMRA}–$CD27^{high}$

Surface phenotype: $CD8^{high}$ $CCR7^{-}$ $CD45RA^{+}$ $CD27^{high}$ $CD28^{-}$



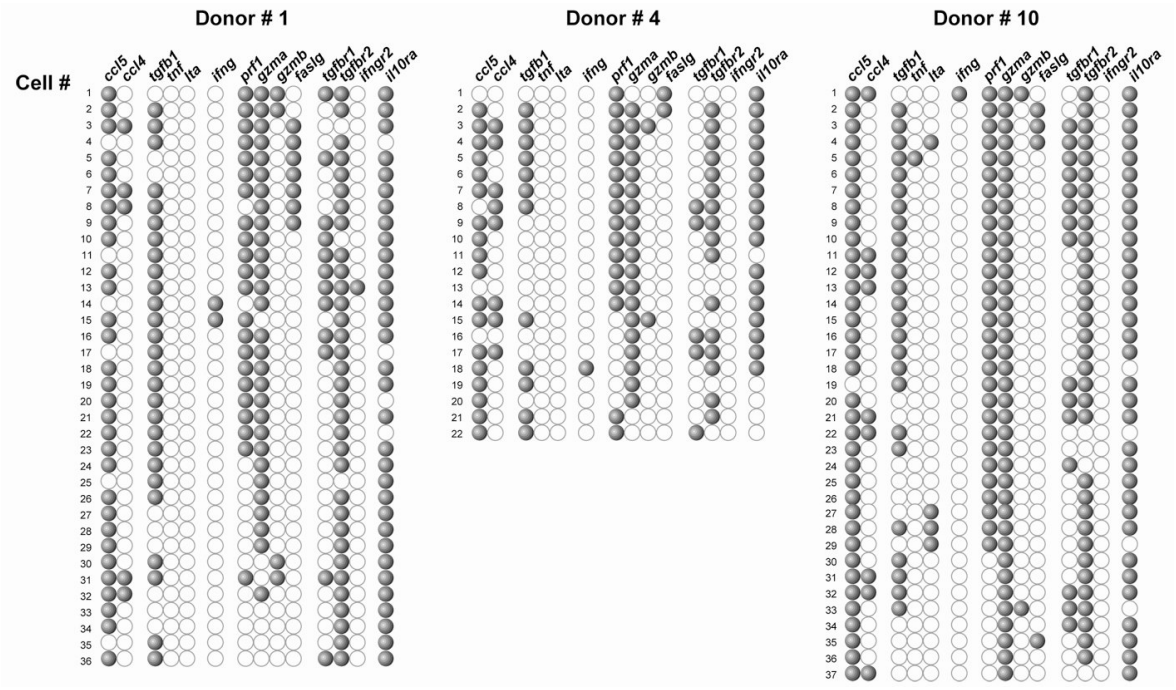
Central Memory (T_{CM})

Surface phenotype: $CD8^{high}$ $CCR7^{+}$ $CD45RA^{-}$ $CD27^{+}$ $CD28^{+}$



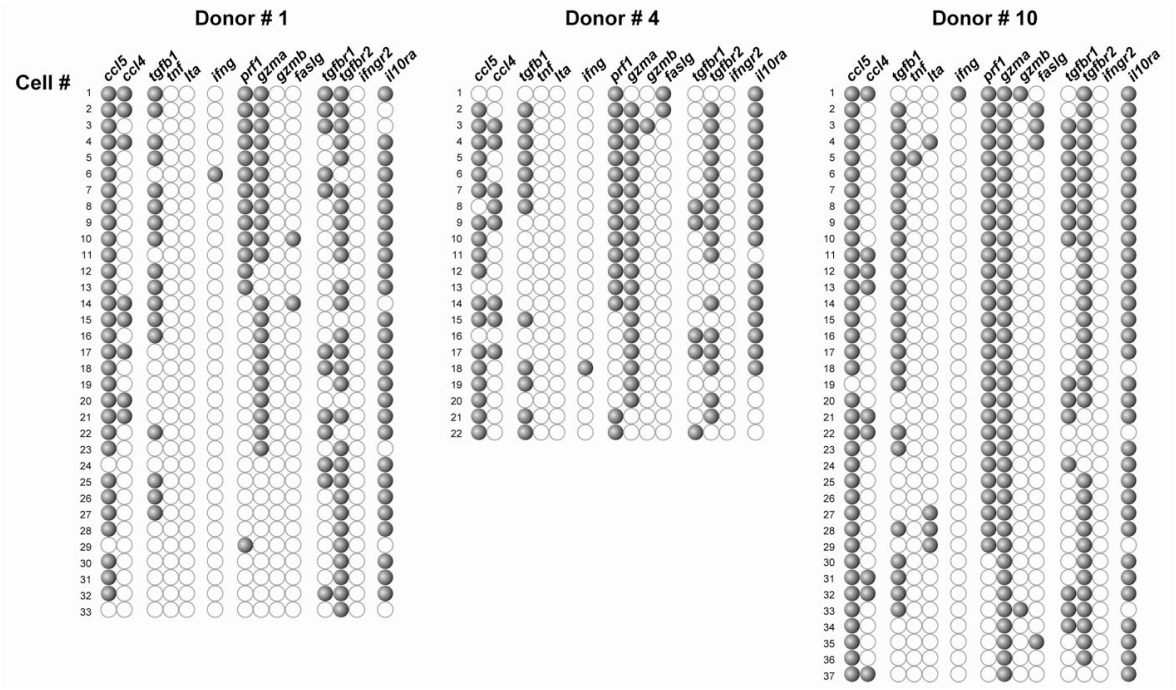
T_{EM}–DP

Surface phenotype: $CD8^{high}$ $CCR7^{-}$ $CD45RA^{-}$ $CD27^{+}$ $CD28^{+}$



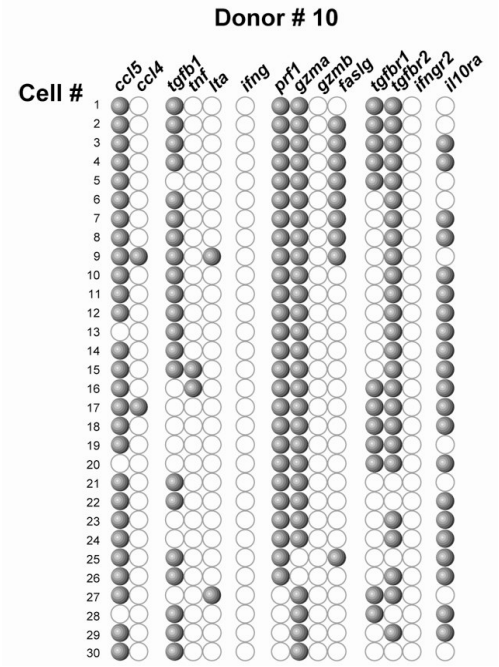
T_{EMRA}–DP

Surface phenotype: $CD8^{high}$ $CCR7^{-}$ $CD45RA^{+}$ $CD27^{+}$ $CD28^{+}$



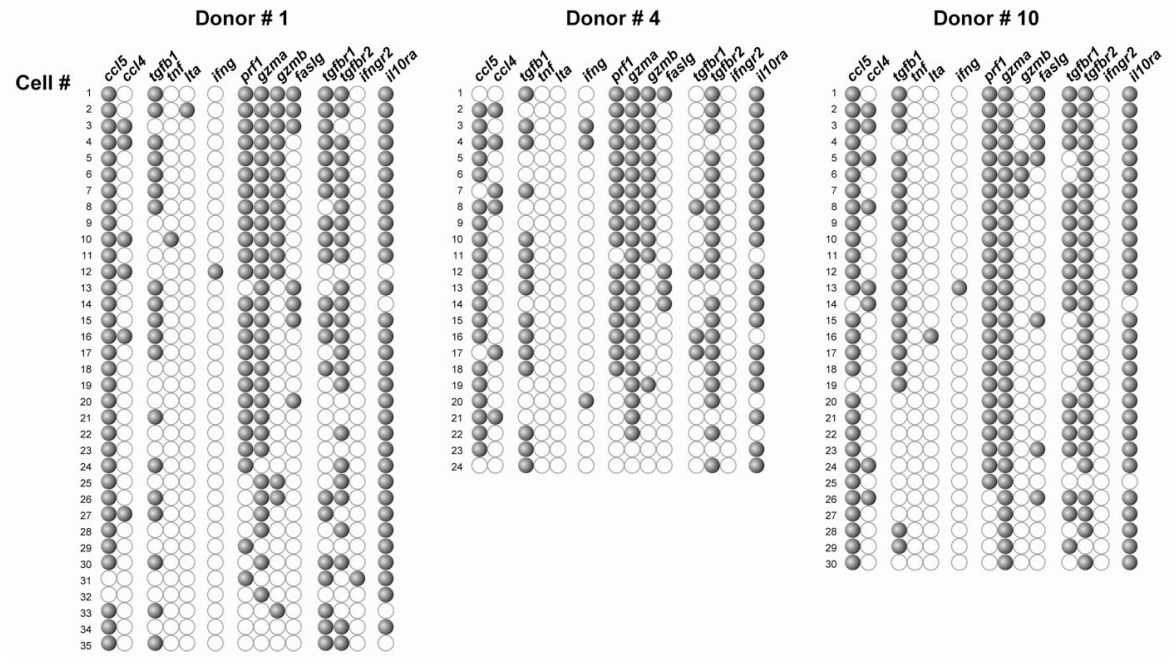
T_{EM}–28SP

Surface phenotype: $CD8^{high}$ $CCR7^{-}$ $CD45RA^{-}$ $CD27^{-}$ $CD28^{+}$



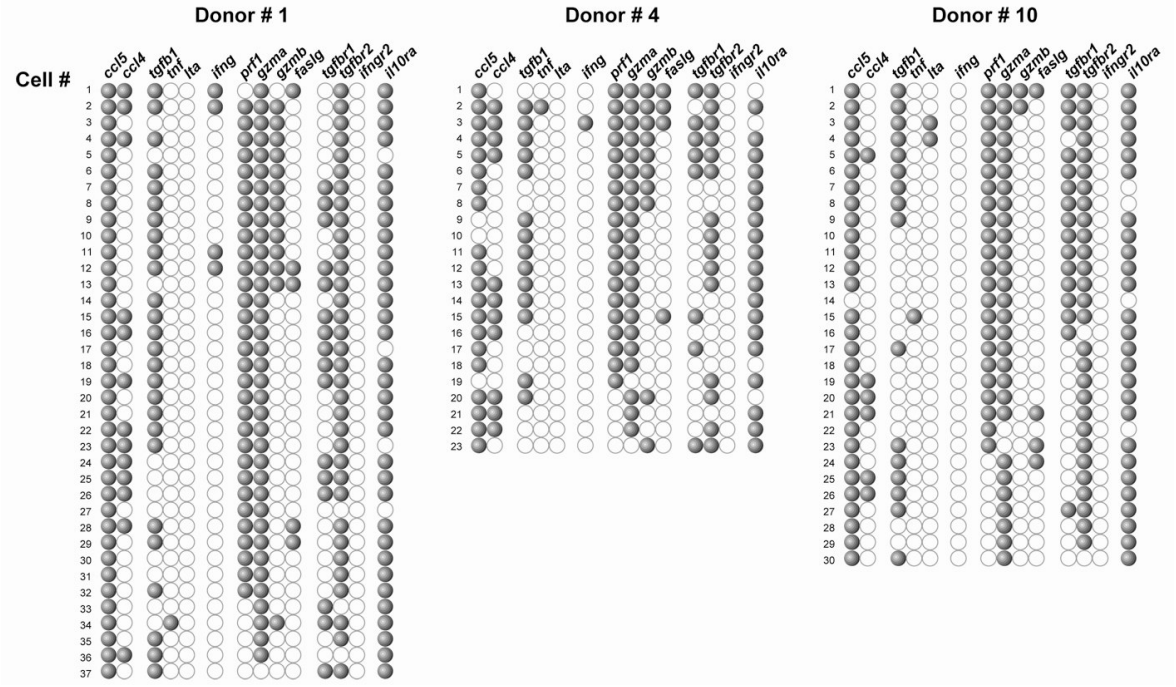
T_{EM}–27SP

Surface phenotype: $CD8^{high}$ $CCR7^{-}$ $CD45RA^{-}$ $CD27^{+}$ $CD28^{-}$



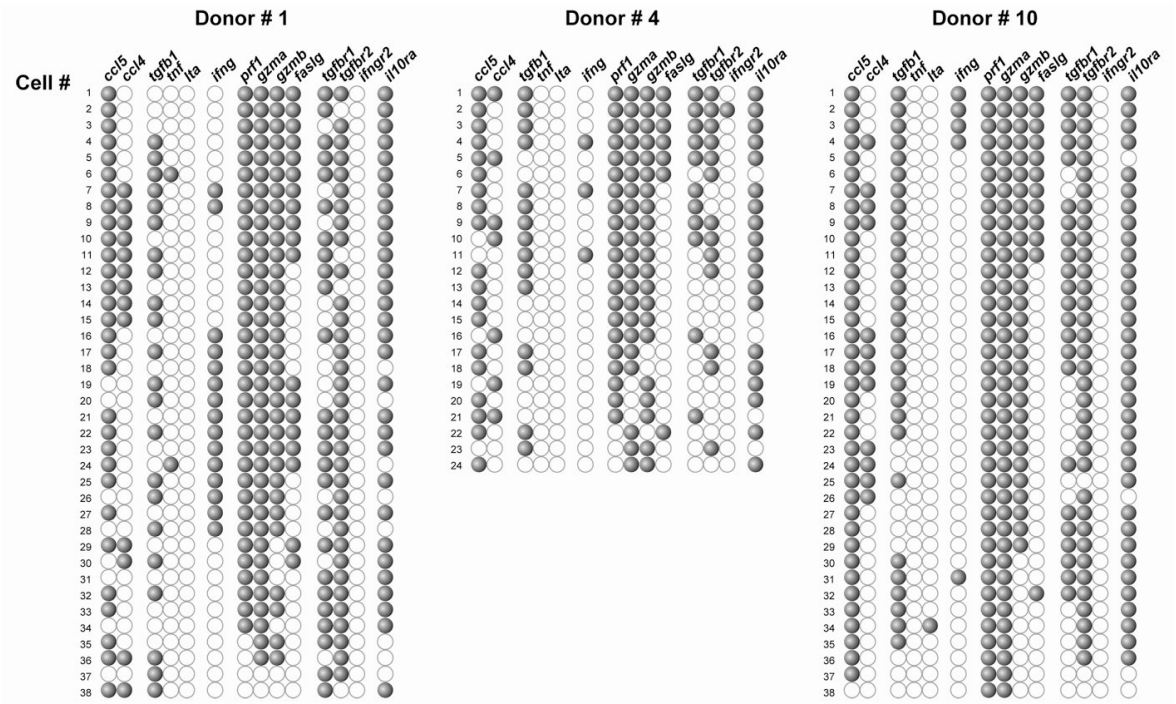
T_{EMRA}–27SP

Surface phenotype: $CD8^{high}$ $CCR7^{-}$ $CD45RA^{+}$ $CD27^{+}$ $CD28^{-}$



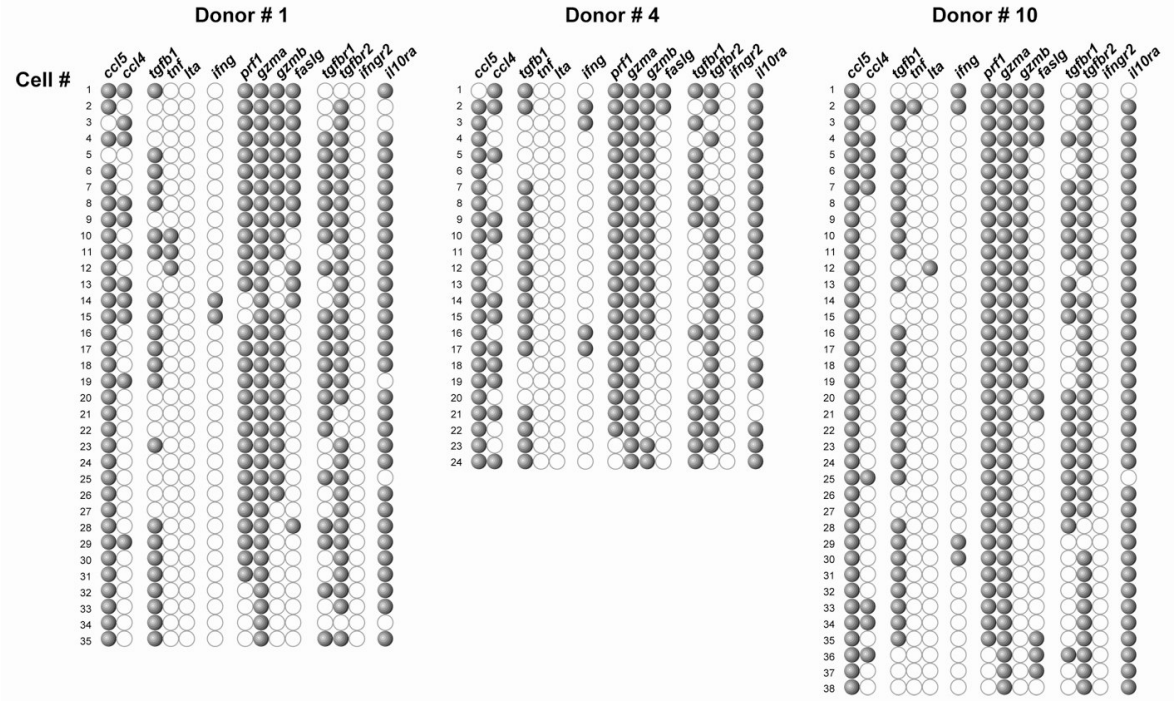
T_{EM}–DN

Surface phenotype: $CD8^{high}$ $CCR7^{-}$ $CD45RA^{-}$ $CD27^{-}$ $CD28^{-}$



T_{EMRA}–DN

Surface phenotype: $CD8^{high}$ $CCR7^{-}$ $CD45RA^{+}$ $CD27^{-}$ $CD28^{-}$



Supplemental Material of Published Manuscript

Supplemental Materials I- Mouse Multiplex

Gene	Acession number	1st PCR Primers	2nd PCR Primers
Prf-1	***ENSMUSG00000037202***	5'-TCACACTGCCAGCGTAATGT-3' 5'-CTGTGGTAAGCATGCTCTGT-3'	5'-CACAGTAGAGTGTCGCATGT-3' 5'-CTGTGGTAAGCATGCTCTGT-3'
Gzma	***ENSMUSG00000023132***	5'-TCAAATACCATCTGTGCTGG-3' 5'-AGAGGGAGCTGACTTATTGC-3'	5'-GGGATCTACAACTTGTACGG-3' 5'-AGAGGGAGCTGACTTATTGC-3'
Gzmb	***ENSMUSG00000015437***	5'-GTCAATGTGAAGCCAGGAGA-3' 5'-AGGATCCGATGTTGCTTCTG-3'	5'-GGGAGTGTGAGTCCTACTTT-3' 5'-AGGATCCGATGTTGCTTCTG-3'
Tnfsf-6	***ENSMUSG00000000817***	5'-TTCATGGTTCTGGTGGCTCT-3' 5'-GAGCGGTTCCATATGTGTCT-3'	5'-TGTATCAGCTCTTCCACCTG-3' 5'-GAGCGGTTCCATATGTGTCT-3'
Ifn-γ	***ENSMUSG00000055170***	5'-GCTCTGAGACAATGAACGCT-3' 5'-AAAGAGATAATCTGGCTCTGC-3'	5'-TGTTTCTGGCTGTTACTGCC-3' 5'-AAAGAGATAATCTGGCTCTGC-3'
Lt-α	***ENSMUSG00000024402***	5'-AGCACAGAAAGCATGATCCG-3' 5'-AACCTGGGAGTAGACAAGGT-3'	5'-CCTCCCTCTCATCAGTTCTA-3' 5'-AACCTGGGAGTAGACAAGGT-3'
Tgf-β	***ENSMUSG00000002603***	5'-ACCATCCATGACATGAACCG-3' 5'-CAATCATGTTGGACAACTGC-3'	5'-GCTACCATGCCAACTTCTGT-3' 5'-CAATCATGTTGGACAACTGC-3'
Tgf-βR1	***ENSMUSG00000007613***	5'-TGTCTCAGTCACTGAGACCA-3' 5'-AGGTGAATGACAGTGCGGTT-3'	5'-TGCAATCAGGACCACTGCAA-3' 5'-AGGTGAATGACAGTGCGGTT-3'
Tgf-βR2	***ENSMUSG00000032440***	5'-AGATGCATCCATCCACCTAA-3' 5'-TGCACTCTTCCATGTTACAG-3'	5'-CGATGTGAGACTGTCCACTT-3' 5'-TGCACTCTTCCATGTTACAG-3'
Tgf-βR3	***ENSMUSG00000029287***	5'-GAGTGAACGTCCATGCACG-3' 5'-TGACTGACAGGGCGATATTC-3'	5'-CATTGGACAATGGCTACAGC-3' 5'-TGACTGACAGGGCGATATTC-3'
IL10R	***ENSMUSG00000032089***	5'-AACAGTCAGTACTCCAACT-3' 5'-CTGCTCCGTCGTGATAAGTA-3'	5'-CGGCATCATCTATGGGACAA-3' 5'-CTGCTCCGTCGTGATAAGTA-3'
IL2	***ENSMUSG00000027720***	5'-TCAAGTCCAGCGTAATGT-3' 5'-CTGTGGTAAGCATGCTCTGT-3'	5'-CTCTACAGCGGAAGCACAGC-3' 5'-CTGTGGTAAGCATGCTCTGT-3'
IL21	***ENSMUSG00000027718***	5'-GATCGCCTCCTGATTAGACT-3' 5'-CTTCGGGTCCTATGTGTTCT-3'	5'-CACTGTGAGCATGCAGCTTT-3' 5'-CTTCGGGTCCTATGTGTTCT-3'
IL21R	***ENSMUSG00000030745***	5'-TCAAGTCCAGCGTAATGT-3' 5'-CTGTGGTAAGCATGCTCTGT-3'	5'-CTCTACAGCGGAAGCACAGC-3' 5'-CTGTGGTAAGCATGCTCTGT-3'
IL15	***ENSMUSG00000031712***	5'-CTTGCAGTGCATCTCCTTAC-3' 5'-AATGCCCAGGTAAGAGCTTC-3'	5'-AAGCACTGCCTCTTCATGGT-3' 5'-AATGCCCAGGTAAGAGCTTC-3'
IL15R	***ENSMUSG00000023206***	5'-ATTGAGCATGCTGACATCCG-3' 5'-TGTGGTCATTGCGGTATCTG-3'	5'-GGTATGTCTGTAACTCTGGC-3' 5'-TGTGGTCATTGCGGTATCTG-3'
IL7R	***ENSMUSG00000003882***	5'-GAGTCCAAGTCCTACCTTCG-3' 5'-CGGTTTGCACTGTGTACAGC-3'	5'-AACCTGTCGTATGGCCTAGT-3' 5'-CGGTTTGCACTGTGTACAGC-3'
CCR7	***ENSMUSG00000037944***	5'-CTAGCTGGAGAGAGACAAGA-3' 5'-TATCCGTCATGGTCTTGAGC-3'	5'-TACGAGTCGGTGTGCTTCAA-3' 5'-TATCCGTCATGGTCTTGAGC-3'
CD3-ε	***ENSMUSG00000032093***	5'-ACCAGTGTAGAGTTGACGTG-3' 5'-TATGGCTACTGCTGTCAGGT-3'	5'-GCTACTACGTCTGCTACACA-3' 5'-TATGGCTACTGCTGTCAGGT-3'
28S (Mrp-S21)	***ENSMUSG00000015746***	5'-CTCTGAAGTTCATTGCCAGG-3' 5'-AGTAACAGAACTTGGCTGGA-3'	5'-CTCTGAAGTTCATTGCCAGG-3' 5'-AGTAACAGAACTTGGCTGGA-3'
Hprt	***ENSMUSG00000025630***	5'-TTCTTTGCTGACCTGCTGGA-3' 5'-ATCCAACACTTCGAGAGGTC-3'	5'-GGTGGAGATGATCTCTCAAC-3' 5'-ATCCAACACTTCGAGAGGTC-3'

Table Supplemental Materials. Specific primers used for the first and second PCR. Primers on the top: 5' primers; Primers on the bottom: 3' primers. Reverse transcription reactions were carried out using the 3' primer indicated for each individual gene. Acession numbers correspond to gene sequences obtained at the Ensembl Project Homepge (http://www.ensembl.org).

Supplemental Materials II- Human Multiplex

Gene	Acession number	1st PCR Primers	2nd PCR Primers
Ccl-5	***ENSG00000161570***	5'-CCTGCTGCTTTGCCTACATT-3' 5'-TCCCAAAGTGCTGGGATTAC-3'	5'-GTGCCCACATCAAGGAGTAT-3' 5'-TCCCAAAGTGCTGGGATTAC-3'
Ccl-4	***ENSG00000129277***	5'-CTCACCTCTGAGAAAACCTC-3' 5'-GATCAGCACAGACTTGCTTG-3'	5'-TACCATGAAGCTCTGCGTGA-3' 5'-GATCAGCACAGACTTGCTTG-3'
Ccl-3	***ENSG00000006075***	5'-ACCTGCTCAGAATCATGCAG-3' 5'-TTCTGGACCCACTCCTCACT-3'	5'-CTCTCTGCAACCAGTTCTCT-3' 5'-TTCTGGACCCACTCCTCACT-3'
Gzma	***ENSG00000145649***	5'-CTCCTCATTCAAGACCCTAC-3' 5'-CACATGGTTCCTGGTTTCACA-3'	5'-CTGCAGCTCACTGTAACTTG-3' 5'-CACATGGTTCCTGGTTTCACA-3'
Gzmb	***ENSG00000100453***	5'-CTTCCTGATACGAGACGACT-3' 5'-CTTGTTGCTAGGTAGCCTGA-3'	5'-CCAGCAGTTTATCCCTGTGA-3' 5'-CTTGTTGCTAGGTAGCCTGA-3'
Pfp-1	***ENSG00000180644***	5'-CCCTCTGTGAAAATGCCCTA-3' 5'-GGAGTGTGTACCACATGGAA-3'	5'-ACCAGCAATGTGCATGTGTC-3' 5'-GGAGTGTGTACCACATGGAA-3'
CD3-ε	***ENSG00000160585***	5'-GGTTATTATGTCTGCTACCC-3' 5'-GGTCAGATGCGTCTCTGATT-3'	5'-TGGAGATGGATGTGATGTCG-3' 5'-GGTCAGATGCGTCTCTGATT-3'
IL2	***ENSG00000109471***	5'-CTCACCAGGATGCTCACATT-3' 5'-ACAATGGTTGCTGTCTCATC-3'	5'-AACCTCTGGAGGAAGTGCTA-3' 5'-ACAATGGTTGCTGTCTCATC-3'
IL10R	***ENSG00000110324***	5'-CCTAGAGATCCACAATGGCT-3' 5'-TGCACTCCTCTTTAGACCAC-3'	5'-CGGGAAGATTCAGCTACCCA-3' 5'-TGCACTCCTCTTTAGACCAC-3'
IL10	***ENSG00000136634***	5'-TGAAGGATCAGCTGGACAAC-3' 5'-CACGGCCTTGCTCTTGTTT-3'	5'-AGCCTTGTCTGAGATGATCC-3' 5'-CACGGCCTTGCTCTTGTTT-3'
Tgf-βR1	***ENSG00000106799***	5'-CCGTGAGGCAGAGATTTATC-3' 5'-TGCCAGTCCTAAGTCTGCAA-3'	5'-CAATGGTACTTGGACTCAGC-3' 5'-TGCCAGTCCTAAGTCTGCAA-3'
Tgf-βR2	***ENSG00000163513***	5'-ACACTAGAGACAGTTTGCCA-3' 5'-GCTGATGCCTGTCACTTGAAA-3'	5'-TGGAAGATGCTGCTTCTCCA-3' 5'-GCTGATGCCTGTCACTTGAAA-3'
Af-1	***ENSG00000159128***	5'-CGACAGTAAATGGTTCACGG-3' 5'-GGTATCAGCGATGTCAAAGG-3'	5'-CACAGATCACAGCAACAGAG-3' 5'-GGTATCAGCGATGTCAAAGG-3'
TNF-α	***ENSG00000111956***	5'-CTCTTCTCCTTCCTGATCGT-3' 5'-CTGGGAGTAGATGAGGTACA-3'	5'-CTCTCTCTAATCAGCCCTCT-3' 5'-CTGGGAGTAGATGAGGTACA-3'
Lt-α	***ENSG00000173503***	5'-ACACCACCTGAACGTCTCTT-3' 5'-GGAAGGCACGGTCCGTGTTT-3'	5'-TGTTGGCCTCACACCTTCA-3' 5'-GGAAGGCACGGTCCGTGTTT-3'
Ifn-γ	***ENSG00000111537***	5'-CTGTTACTGCCAGGACCCAT-3' 5'-TGGATGCTCTGGTCATCTTT-3'	5'-GGTCATTCAGATGTAGCGGA-3' 5'-TGGATGCTCTGGTCATCTTT-3'
Tgf-β	***ENSG00000105329***	5'-GACAAGTTCAAGCAGAGTACA-3' 5'-CACAACTCCGGTGACATCAAA-3'	5'-ACACATCAGAGCTCCGAGAA-3' 5'-CACAACTCCGGTGACATCAAA-3'
Tnfsf-6	***ENSG00000117560***	5'-ACACATCAGAGCTCCGAGAA-3' 5'-GGCAGGTTGTTGCAAGATTG-3'	5'-CAGCTCTTCCACCTACAGAA-3' 5'-GGCAGGTTGTTGCAAGATTG-3'
CCR7	***ENSG00000126353***	5'-CCTTCCTGTGTGGTTTTACC-3' 5'-CATGGTCTTGAGCCTCTTGA-3'	5'-TGGTGGCTCTCCTTGTCATT-3' 5'-CATGGTCTTGAGCCTCTTGA-3'

Table Supplemental Materials. Specific primers used for the first and second PCR. Primers on the top: 5' primers; Primers on the bottom: 3' primers. Reverse transcription reactions were carried out using the 3' primer indicated for each individual gene. Acession numbers correspond to gene sequences obtained at the Ensembl Project Homepge (http://www.ensembl.org).

LIST OF PUBLICATIONS

PEER REVIEWD PUBLICATIONS

Monteiro M, Evaristo C, Legrand A, Nicoletti A, Rocha B.
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